PROPERTIES OF NAX-5055: A NOVEL SYSTEMICALLY AVAILABLE GALANIN-BASED NEUROPEPTIDE

by

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ABSTRACT

Epilepsy describes a spectrum of common and devastating neurological disorders characterized by recurrent unprovoked seizures. Approximately 70% of epilepsy patients achieve seizure freedom with the use of clinically available antiseizure drugs, while the remaining 30% of patients are considered drug-resistant. It has been proposed that in order to effectively treat drug-resistant patients, future research must focus on novel molecular targets that are capable of modifying the underlying pathology of epilepsy.

Potential novel targets include the neuropeptide galanin and its cognate receptors. Hippocampal galanin expression is increased following seizure activity and experimentally increasing central galanin produces antiseizure activity in a number of animal models. However, the specific role of the galanin receptors (GalR1-3) in mediating the anti-seizure properties of galanin remains unclear. To address this issue a collaboration was established between the laboratories of Drs. H. Steve White and Grzegorz Bulaj to design, synthesize, and characterize novel, receptor-preferring, galanin-based neuropeptides. One analog generated from these studies, NAX-5055, displays a 15-fold preference for the GalR1 receptor and potent antiseizure activity in a battery of seizure and epilepsy models.

The overall goal of this dissertation was to further characterize the properties of NAX-5055. Electrophysiological studies evaluated the mechanism of action of NAX-5055. Resutlts from these studies demonstrate an inhibition of presynaptic glutamate

release in the CA3 region of the hippocampus. Interestingly, behavioral studies demonstrated that the antiseizure efficacy of NAX-5055 was markedly reduced following repeated systemic administration. As a result, we investigated the potential mechanism(s) that could account for this observation. Our studies suggest that the reduced efficacy of NAX-5055 is not likely due to an alteration in galanin receptor function, efflux transport by P-glycoprotein, or increased peripheral metabolism.

The studies presented in this dissertation were able to rule out several candidate mechanisms for the reduced efficacy of NAX-5055; however, additional work will be required to fully understand this phenomenon. In addition, our functional studies provide the first indication that galanin can decrease hippocampal excitability through a presynaptic mechanism. Future studies with NAX-5055 will increase our understanding of galanin and its receptors *in vivo* and help guide the development of future neuropeptide-based theraputics.

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CHAPTER 1

INTRODUCTION

Neuropeptides are a large group of signaling molecules expressed throughout the central and peripheral nervous systems. Following repetitive neuronal stimulation, neuropeptides can be released into the extracellular space and modulate surrounding synaptic activity through interaction with pre- and post-synaptic G-protein-coupled receptors. The modulatory activity of these molecules has been implicated in the pathophysiology of a number of neurological disorders that include epilepsy, Alzheimer's disease, and neuropathic pain. As a result there has been significant interest in the development of neuropeptide-based therapeutics (Malavolta and Cabral, 2011; Robertson et al., 2011). In the following dissertation, I describe the functional and behavioral activity of a neuropeptide galanin analog, NAX-5055, with a specific focus on its antiseizure properties.

Epilepsy and Therapeutic Intervention

Epilepsy affects more then 50 million people worldwide; making it one of the world's most common neurological disorders (WHO, 2007). Within the United States, there are approximately 2.2 million people living with epilepsy and an estimated 150,000 new cases diagnosed annually (Hirtz et al., 2007; Institute of Medicine, 2012). Epilepsy

is characterized by the development of spontaneous recurrent seizures, which can manifest in a variety of electrical and behavioral phenotypes. In general, seizures can be defined as temporally distinct, paroxysmal ictal discharges within specific (i.e., focal seizure) or widespread brain regions (i.e., generalized seizure) (D'Ambrosio and Miller, 2010). The neuroanatomical location of the seizure focus can vary in patients with different seizure disorders. For example, in temporal lobe epilepsy, a common seizure disorder, the seizure focus is often found in the hippocampus which can be characterized by hippocampal sclerosis, and can often by treated by the resection of this brain region (Falconer, 1974; Engel, 1996; Engel et al., 2003). As a result, clinical and experimental epilepsy research has centered its attention on the hippocampus as a region of interest to understand the pathology of epilepsy and to test potential therapeutic interventions (O'Dell et al., 2012).

Drugs administered for the treatment of epilepsy have classically been defined as antiepileptic drugs (AED). However, many of these drugs only inhibit seizure activity without affecting the overall progression of the disease state. As a result these drugs are more correctly termed anti-seizure drugs (Galanopoulou et al., 2012). The term antiepilepsy therapy has thus been reserved for drugs that prevent or delay the progression of epilepsy (Galanopoulou et al., 2012). To date, there are no dugs clinically available that are considered true antiepilepsy therapies.

There are currently over 20 antiseizure drugs available with varying mechanisms of action. These include drugs that decrease neuronal excitability through inhibition of voltage-gated Na^+ channels or increase inhibitory tone through augmentation of GABAergic neurotransmission. These drugs continue to provide the clinician and patient

with a broad range of treatment options (Bialer and White, 2010). Pharmacological treatment with antiseizure drugs results in seizure freedom for approximately 70% of epilepsy patients; many are effectively controlled by mono-therapy (Stephen and Brodie, 2012). However, the remaining 30% of epilepsy patients do not achieve full therapeutic control of their seizures and are thus considered therapy resistant (Löscher and Schmidt, 2011). Therapy-resistant epilepsy is clinically defined as a failure to achieve seizure control following treatment with two well-tolerated, appropriately chosen anti-seizure drugs administered as mono-therapy or in combination (Kwan et al., 2010). Surgical intervention provides an additional and effective treatment option for some drug-resistant patients, but requires the identification of a clear seizure focus that can be easily resected (Engel, 1996; Engel et al., 2003). As a result, there remains a significant population of therapy-resistant epilepsy patients who are not candidates for surgical resection and are thus left with limited options for the treatment of their seizures (Cascino, 2008). It has been suggested that in order to help these patients, the focus of therapeutic intervention should shift toward modulation of the pathological brain, not simply decreasing neuronal excitability (Löscher and Schmidt, 2011). Thus, there has been considerable interest in novel molecular targets aimed at modulating pathological synaptic transmission.

Neuropeptides as Drug Targets for Epilepsy

The neuropeptides and their receptors present interesting targets for the development of novel therapeutic interventions for patients with epilepsy (Robertson et al., 2011). Neuropeptides are a class of signaling molecules distinct from classical neurotransmitters in size, synthesis, and mechanism of action. Neuropeptides are

synthesized from larger precursor proteins in the cell soma and packaged into dense core vesicles (DCV) that require axonal transport to reach presynaptic terminals. Modulation of neuropeptide levels within the presynaptic terminal is relatively slow (i.e., minutes to hours) and requires changes in gene expression and additional DCV trafficking. Unlike synaptic vesicles, DCV release is not restricted to the active zone of the presynaptic terminal. Furthermore, neuropeptides are not released following single action potential depolarization-induced increases in Ca^{2+} microdomains at the active zone. Thus, during periods of low neuronal firing, neuropeptides have little influence on synaptic activity. However, high frequency neuronal firing, similar to that observed during seizure activity, increases Ca^{2+} levels throughout the presynaptic terminal to concentrations sufficient to induce DCV vesicle fusion and neuropeptide release. Neuropeptide signaling is mediated by volume transmission thus allowing for modulation of synaptic activity in a large area surrounding the site of release. In addition, neuropeptides are not subject to active reuptake, but rather are inactivated enzymatically or by diffusion. This results in a relatively long duration of activity. Thus, the neuropeptides are well suited to facilitate or inhibit highly active neuronal circuits. Indeed, following intense seizure activity, a number of neuropeptides, including galanin, neuropeptide Y and somatostatin, are released within the hippocampus and act to facilitate or inhibit seizure activity (Robertson et al., 2011). The work presented in this dissertation is focused on the anti-seizure activity of the neuropeptide galanin.

Galanin Regulation of Seizure Activity

Galanin is a 29 (30 in humans) amino acid neuropeptide originally isolated from the porcine intestine (Tatemoto et al., 1983). It displays extensive expression in the central, peripheral, and enteric nervous system (Melander et al., 1986). The first 15 N-terminal amino acids of galanin are highly conserved between species, including humans (Evans and Shine, 1991). Galanin is synthesized by proteolytic processing of the 123-amino acid preprogalanin precursor protein (Kofler et al., 1996). Along with galanin, a 59-amino acid peptide known as galanin message associated peptide (GMAP) is synthesized. Unlike galanin, GMAP does not display binding affinity for any of the known galanin receptors (Rökaeus and Brownstein, 1986; Lang et al., 2007).

The first suggestion that galanin could regulate seizure activity was the demonstration of its expression within the hippocampal formation; a common seizure focus in temporal lobe epilepsy patients. There are two major galanin-containing projections that innervate the hippocampus; one a noradrenergic projection originating from the locus coeruleus and the second a cholinergic projection originating in the septum/diagonal band complex (Melander et al., 1986; Consolo et al., 1994; Xu et al., 1998). These galanin-containing projections extend throughout the hippocampal formation, but appear particularly robust in the ventral regions (Yoshitake et al., 2011). Galanin projections are also of particularly high density within the dentate gyrus (Mazarati, 2004). This is an interesting observation given that the dentate gyrus is thought to serve as a gate for the propagation of seizure activity within the hippocampus (Heinemann et al., 1992; Coulter and Carlson, 2007). It is also interesting to note that *de novo* neuronal galanin expression is observed following seizure activity in the hilar

region of the dentate gyrus (Mazarati et al., 1998). It has been hypothesized that upregulation of galanin in the hilar region is specific to interneurons and occurs as an compensatory anti-seizure response (Mazarati et al., 1998). Although this is an intriguing hypothesis, further testing is required to confirm these observations.

Mazararati et al. (1992) were the first to provide direct evidence that galanin could inhibit seizure activity in the brain. In their studies, galanin was shown to decrease the severity of picrotoxin-kindled seizures in rats following bilateral injection (50-200 ng) into the lateral ventricles (Mazarati et al., 1992). Later, the same group provided compelling evidence of the antiseizure properties of galanin specifically within the hippocampus. Their studies utilized perforant path stimulation and Li-pilocarpine models of status epilepticus (SE) in which animals experienced a prolonged bout of severe seizure activity followed by the development of spontaneous seizures. Direct injection of galanin (0.5 nmol) into the hilar region of the dentate gyrus inhibited the induction of SE and blocked seizure activity in rats with previously established SE (Mazarati et al., 1998). Further, the effects of galanin on SE were attenuated by the nonselective galanin receptor antagonist M35 (Mazarati et al., 1998). Immunohistochemical evaluation of the hippocampus following SE illustrated that galanin staining is nearly abolished three hours after the initiation of seizure activity; suggesting exhaustion of galanin stores (Mazarati et al., 1998; Lerner et al., 2008).

Under normal physiological conditions galanin, like many peptides, is degraded by endogenous peptidases once it is released, making it difficult to study the function of long-term activation. As a result, a number of investigators have generated genetically modified mice that show increased or decreased galanin expression. Overexpression of

galanin, under the dopamine-β-hyroxylase promoter, attenuated the severity of SE induced by either perforant path stimulation or the glutamate agonist kainic acid (KA) (Mazarati et al., 2000). More widespread overexpression of galanin was achieved using the platelet-derived growth factor B (PDGF) promoter and resulted in delayed kindling acquisition in both mice and rats (Kokaia et al., 2001; Schlifke et al., 2006). In contrast, in galanin knockout mice the seizure phenotype was exaggerated, such that animals were more susceptible to seizure induction and cell death (Kerr et al., 2000; Mazarati et al., 2000).

The success of maintaining the antiseizure properties of galanin through genetic overexpression has made galanin a target for the development of gene therapy approaches. A number of elegant studies have utilized adeno-associated virus (AAV) vectors to increase central galanin levels. The first of these studies used a fibronectin secretory sequence (FIB) to ensure the active secretion of galanin into the extracellular space (Haberman et al., 2003). Injection of AAV-FIB-galanin into the inferior colliculus attenuated seizures following electrical stimulation, whereas injection into the piriform cortex attenuated seizures induced by KA treatment (Haberman et al., 2003; McCown, 2006). Utilizing the neuron-specific promoter neuron specific enolase (NSE), direct injection of AAV-NSE-galanin into the dentate gyrus blocked the development of seizures following an intrahippocampal KA injection (Lin et al., 2003). AAV-NSE-galanin was also shown to delay the progression of kindled seizures by slowing the development of kindling; a finding consistent with that observed following overexpression with PDGF (Kanter-Schlifke et al., 2007).

One proposed mechanism for the antiseizure activity of galanin is the inhibition of neurotransmitter release. Incubation of transverse hippocampal brain slices with galanin inhibits the release of glutamate and aspartate following a depolarizing stimulation (Zini et al., 1993). Similar studies have been conducted utilizing transgenic mice. In hippocampal slices taken from mice overexpressing galanin with the dopamine-β-hydroxylase promoter, a significant reduction in the release of glutamate following slice depolarization was observed (Mazarati et al., 2000). However, in hippocampal slices taken from galanin-knockout mice a significant increase in glutamate release following slice depolarization was reported (Mazarati et al., 2000). Although these studies strongly suggest that galanin is an effective regulator of hippocampal excitability, it still remains unclear what cellular mechanisms mediate the observed inhibition of glutamate release.

Role of Galanin Receptors in Seizure Regulation

There have been three galanin receptors cloned to date, GalR1-3; all belong to the G-protein coupled receptor super family. All three receptor subtypes are expressed in the brain, but only GalR1 and GalR2 have been shown to be expressed in the hippocampus. It is still unclear how each receptor mediates the anti-seizure activity of galanin. Understanding the localization of the galanin receptors has relied heavily on the use of *in situ* hybridization and autoradiography due to unreliable antibodies and limited receptor-selective pharmacology (Lu and Bartfai, 2009).

GalR1 Receptors

The human GalR1 receptor was originally cloned from the Bowes melanoma cell line; whereas the rat version of the GalR1 receptor was cloned from rat brain and Rin13B insulinoma cells (Habert-Ortoli et al., 1994; Burgevin et al., 1995; Parker et al., 1995). There is 92% similarity between the human, rat, and mouse GalR1 receptor homologs (Jacoby et al., 1997; Branchek et al., 2000). The GalR1 receptor is expressed throughout the body, including the gastrointestinal tract, pancreas, brain, and spinal cord (Gustafson et al., 1996; Pham et al., 2002; Hohmann et al., 2003; Barreto et al., 2011). Within the brain, the expression pattern is broad and includes the amygdala, lateral septum, ventral hippocampus, and thalamus (Gustafson et al., 1996; Hohmann et al., 2003; Jungnickel and Gundlach, 2005). When expressed in immortalized cell lines GalR1 has been shown to inhibit forskolin-stimulated cAMP release, open G-protein-coupled inwardly rectifying (GIRK) channels, and stimulate mitogen-activated protein kinase (Habert-Ortoli et al., 1994; Parker et al., 1995; Smith et al., 1998; Wang et al., 1998b). These intracellular pathways are all inhibited in the presence of pertussis toxin; suggesting that the GalR1 receptor selectively couples to a G_i-type G-protein. Following receptor activation in immortalized cell lines, GalR1 receptors are internalized in a clathrin-dependent manner (Wang et al., 1998a; Xia et al., 2008). Once internalized, receptors are trafficked to lysosomes for degradation, which is dependent on a C-terminal signal motif, suggesting a tight regulation of membrane expression for the GalR1 receptor (Xia et al., 2008).

GalR2 Receptors

The second cloned galanin receptor (GalR2) was first cloned in the rat hypothalamus, GalR2 homologs has also been cloned for both mouse and human (Smith et al., 1997; Pang et al., 1998; Fathi et al., 1998a). There is a high level of homology between the GalR2 receptor across species, but only 40% homology with rat and human GalR1 (Howard et al., 1997; Wang et al., 1997). The mRNA expression pattern of GalR2 is also quite broad; appearing in almost all tissues including the brain and spinal cord (Branchek et al., 2000). Within the brain, the GalR2 receptor is expressed in many of the same areas as GalR1 including the amygdala and hippocampus. GalR2 is also found in the hypothalamus and the dentate gyrus (Fathi et al., 1998b; O'Donnell et al., 1999). Unlike GalR1 receptors, the GalR2 receptor has been suggested to interact with multiple G-proteins that may utilize different intracellular cascades. The most commonly reported effect of GalR2 is the activation of phospholipase C (PLC). This results in the release of intracellular Ca^{2+} stores and the opening of calcium-dependent chloride channels (Fathi et al., 1997; Smith et al., 1997; Pang et al., 1998). Activation of this pathway may also result in the inhibition of caspase-3 and caspase-9; effects that may account for the neuroprotective effects of galanin that have been attributed to activation of GalR2 (Hobson et al., 2006; Elliott-Hunt et al., 2007; 2011). These intracellular effects were not affected by pertussis toxin; suggesting an interaction with the $G_{q/11}$-type G-protein. The interaction of GalR2 with the G_i-type G-protein has remained controversial; i.e., some studies have shown an inhibition of forskolin-stimulated cAMP accumulation, whereas others have been unable to repeat these effects in similar cell lines (Fathi et al., 1997; Smith et al., 1997; Wang et al., 1997). In those studies that observe an inhibition of

cAMP accumulation, the effects are pertussis toxin-sensitive, thereby suggesting a potential coupling of GalR2 to the G_i-type G-protein (Wang et al., 1997). Following activation, GalR2 receptors, like the GalR1 receptors, are internalized. However, once internalized the receptor rapidly recycles back to the cell membrane following galanin washout (Xia et al., 2005; Lu et al., 2005c).

Galanin Receptor Regulation of Seizure Activity

The study of galanin receptor subtype specificity of the anti-seizure effects of galanin has been limited somewhat by the lack of receptor-selective pharmacology. As a result, much of what we know about galanin receptors in regulating seizure activity has come from studies with genetically modified mice. The GalR1 knockout mouse was generated on a C57BL/6J background (Jacoby et al., 2002). It has been documented that some of these mice display spontaneous seizures; although this has not been reported in all studies utilizing these mice (Jacoby et al., 2002; Mazarati et al., 2004b; McColl et al., 2006). Mazarati et al. demonstrated that the cumulative time spent in seizures and the single seizure duration were increased in GalR1 knockout mice when SE was induced by Li-pilocarpine or perforant path stimulation, but not KA administration (Mazarati et al., 2004b; Schauwecker, 2010). However, significant neuronal loss was observed in CA1, CA2 and the hilus of GalR1 knockout mice following systemic KA administration. This finding suggests that the GalR1 receptor may also be involved in cell survival (Schauwecker, 2010). Partial transient knockdown of the GalR2 receptors in the hippocampus utilizing peptide nucleic acid antisense oligonucleotides resulted in an increase in severity and duration of seizures following perforant path stimulation

(Mazarati et al., 2004a). However, in a full GalR2 knockout mouse there was no difference in seizure susceptibility following either pentylenetetrazole (PTZ) or flurothyl induced seizures (Gottsch et al., 2005). Based on these studies, it seems likely that both GalR1 and GalR2 are involved in regulating seizure susceptibility; however additional work is required to uncover the mechanism(s) underlying the activity of each receptor type.

Galanin-Receptor Interactions and Pharmacology

Site-directed mutagenesis studies have illustrated a series of critical residues facilitating the interaction of galanin with its receptors. For the GalR1 receptor, the major interactions between receptor and peptide were found to be His^{264} and His^{267} toward Trp^{2}, Phe^{292} toward Try^{9}, and Phe^{115} toward Gly^{1} (Berthold et al., 1997; Church et al., 2002). Studies utilizing the non-GalR1 selective galanin fragment, Gal (2-11) (AR-1896), have identified the key residues facilitating the GalR2-agonist interaction. These residues include His^{252}, His^{253}, Ile^{256}, Phe^{264}, and Tyr^{271} on the GalR2 receptor and Trp^{2}, Asn^{5}, Gly^{8}, Tyr^{9}, and Leu^{10} on the peptide fragment (Lundström et al., 2005; 2007). A number of galanin chimeric peptides and galanin fragments have been generated and characterized. The chimeric peptides including C7, M15, M32, M35, and M40 all maintain nanomolar binding affinity for galanin receptors and demonstrate antagonistic activity (Lang et al., 2007). The full rat galanin, Gal(1-29) and the N-terminal fragment Gal(1-16) demonstrate a slight preference for the GalR1 receptor. The full peptide displays a slightly higher binding affinity. Both full length galanin and Gal(1-16) also display anticonvulsant activity following intracerebroventricular (i.c.v.) injection (Lang

et al., 2007). Perhaps the most useful galanin fragment characterized to date is Gal (2-11) (AR-1896). This particular peptide displays no appreciable affinity for the GalR1 receptor and as such provides a useful pharmacological tool to isolate the activity of GalR2/3 receptors (Lu et al., 2005a).

In addition to these galanin fragments and chimeras two small-molecule receptor ligands have been synthesized: galmic and galnon (Saar et al., 2002). Galnon displaces I^{125}-galanin in rat ventral hippocampus and inhibits forskolin-stimulated cAMP accumulation (Saar et al., 2002). Systemic injection of galnon was found to abolish PTZ-induced seizures in mice; whereas intrahippocampal administration decreased the duration of perforant path-stimulated SE (Saar et al., 2002). Galmic displays micromolar binding for only GalR1 and attenuates perforant path-stimulated SE following both systemic and intrahippocampal administration (Bartfai et al., 2004). Despite the benefits of a large number of pharmacological tools many of these compounds are only effective following central administration because they are susceptible to rapid enzymatic degradation and lack the ability to penetrate the blood brain barrier. Those that are active systemically have relatively low binding affinity. Furthermore, both galmic and galnon display off target activity within their effective concentration range (Lu et al., 2005b).

Bulaj et al. (2008) utilized a combination of cationization and lipidization approaches in an effort to generate a new series of systemically available galanin analogs. The lead analog from these studies, NAX-5055, maintains the Gal(1-13) core of the endogenous galanin peptide with the addition of a palmitoyl fatty acid tail attached to a lysine residue and cationization of the N-terminus (Figure 1.1A) (Bulaj et al., 2008).

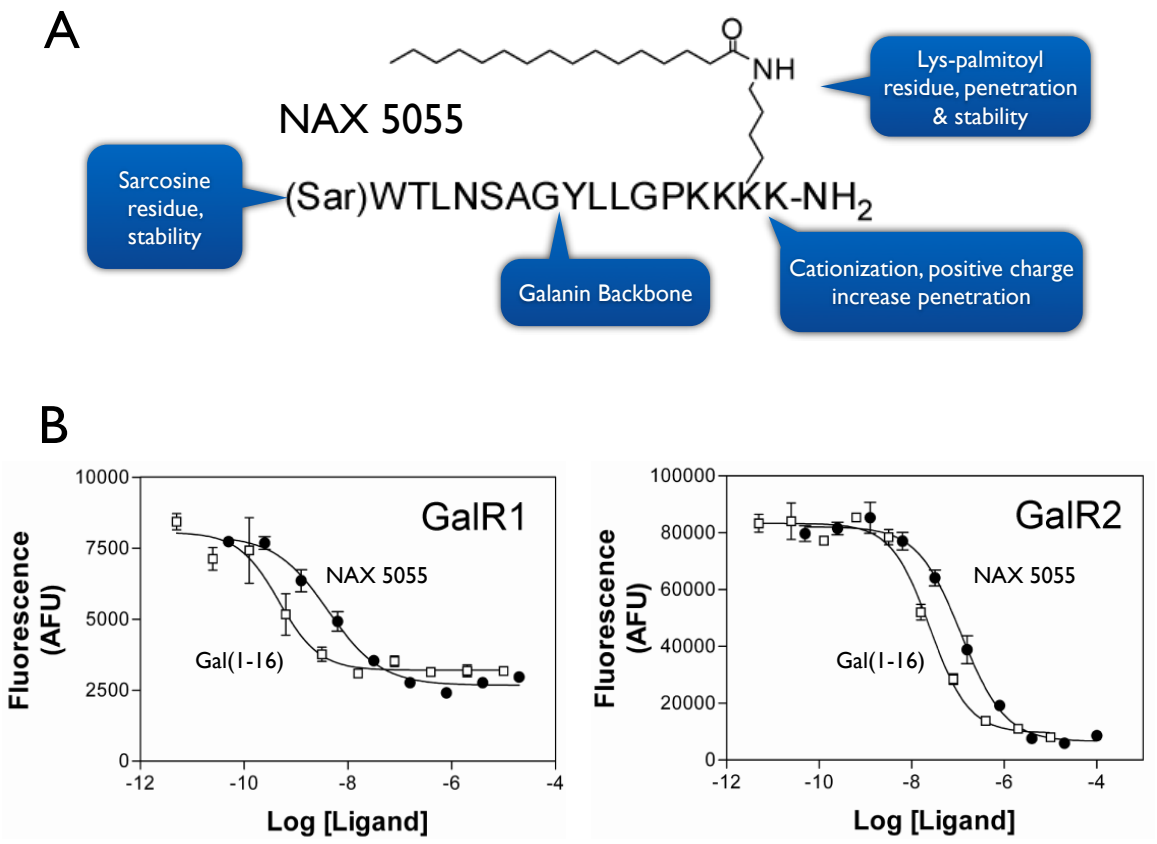

Figure 1.1. Chemical structure and binding properties of the novel galanin analog, NAX-5055

A) Diagram of the chemical structure of NAX-5055 illustrating the backbone galanin fragment, Gal (1-13), as well as the cationization and lipidization modifications added to increase bioavailability.

B.) Time resolved fluorescence competition binding assays of NAX-5055 (●) or the galanin fragment Gal (1-16) (□) incubated with europium tagged galanin (1-29). Binding affinity (K_i) for both Gal (1-16) and NAX-5055 are in the nanomolar range for both GalR1 and GalR2 receptors. Comparison of binding affinity for both galanin receptors demonstrates a 15-fold preference of NAX-5055 for GalR1. Adapted from (Bulaj et al., 2008)

NAX-5055 displays nanomolar affinity for both galanin receptors with a 15-fold preference for GalR1 (i.e. 3.5 nM for GalR1 and 51.5 nM for GalR2) (Figure 1.1B), and demonstrates an increased half life of approximately 10 hrs in an *in vitro* rat serum assay (Bulaj et al., 2008). When administered systemically, NAX-5055 displays minimal motor toxicity and exhibits potent anticonvulsant activity in a battery of animal seizure and epilepsy models (Bulaj et al., 2008; White et al., 2009). This analog thus provides a novel and interesting tool for further addressing the role of galanin receptors in control of seizures.

Dissertation Overview

The goal of this dissertation was to characterize the mechanism of action and anti-seizure properties of the novel galanin analog NAX-5055. The work presented in Chapter 2 begins to elucidate the functional activity of NAX-5055 within the hippocampus. We used whole-cell patch clamp recordings of miniature excitatory postsynaptic currents (mEPSCs) to test the hypothesis that NAX-5055 decreases hippocampal excitability by reducing presynaptic glutamate release. To our knowledge this is the first study to delineate between pre- and post-synaptic mechanisms of a galanin-based neuropeptide.

The second aim of this work was to determine if NAX-5055 was capable of delaying the development of kindled seizures in a manner similar to what has been reported in galanin-overexpressing mice and rats (Kokaia et al., 2001; Kanter-Schlifke et al., 2007). If NAX-5055 could inhibit or delay kindling acquisition, and thus limit disease progression, this peptide could be considered to possess important anti-seizure

and disease modifying properties. However, as presented in Chapter 3, NAX-5055, under the conditions tested, did not affect the acquisition of corneal kindling. This result was unexpected and led to the question of what effect repeated administration of NAX-5055 might have on the efficacy of this peptide. Further studies in the 6 Hz psychomotor seizure model demonstrated that following repeated administration, the anti-seizure efficacy of NAX-5055 was markedly reduced.

I evaluated three different hypotheses that could account for the dramatic reduction in efficacy of NAX-5055 following repeated systemic administration. The first two hypotheses are discussed in detail in Chapter 3. The first hypothesis proposes that central galanin receptors display decreased availability for interaction with NAX-5055 as a result of receptor internalization or decreased sensitivity. The second hypothesis proposes that the efflux transport of NAX-5055 is increased such that therapeutic brain levels are no longer attained. The third and final hypothesis is discussed in Chapter 4 and proposes that an increase in peripheral metabolism of NAX-5055 may reduce the amount of functional peptide available for interaction with central galanin receptors.

The studies presented in this dissertation do not provide a clear mechanism underlying the reduced efficacy following multiple dosing with NAX-5055. However, this work outlines a series of *in vivo* and *in vitro* assays that will be useful in future characterization and validation of the clinical utility of neuropeptide-based theraputics. Together, the studies presented in this dissertation open more questions than they answer, but lay the groundwork for the use of NAX-5055 and other galanin analogs to increase our understanding of the therapeutic potential of galanin and its cognate receptors.

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CHAPTER 2

MODULATION OF HIPPOCAMPAL EXCITATORY SYNAPTIC TRANSMISSION BY THE NOVEL GALANIN AGONIST, NAX-5055

Abstract

Galanin is a 29 amino acid endogenous neuropeptide with a broad expression pattern within the central and peripheral nervous systems that has been suggested to protect against pathological changes in a number of neurological disorders. There are three cloned galanin receptors, GalR1, 2 and 3 that all belong to the G-protein family of receptors. It has been suggested that galanin modulates neuronal excitability within the hippocampus by reducing the probability of glutamate release. However, it is unclear how each receptor subtype mediates this physiological activity of galanin. We have recently designed a systemically active, metabolically stable GalR1-preferring galanin analog, NAX-5055. In this study we sought to determine if NAX-5055 could reduce hippocampal excitatory synaptic transmission by inhibiting presynaptic glutamate release onto CA3 pyramidal neurons. We demonstrate in mouse organotypic hippocampal slice cultures that NAX-5055 (500 nM) increases the inter-event interval of miniature excitatory postsynaptic currents (mEPSC) in CA3 pyramidal cells without having any effect on the amplitude of these events. These findings suggest that activation of GalR1

receptors on presynaptic glutamatergic terminals in CA3 results in a decrease in the probability of glutamate release from these terminals. This is the first physiological evidence suggesting that GalR1 receptors are located on presynaptic glutamatergic terminals in CA3 and that this receptor subtype can modulate presynaptic glutamate release. NAX-5055 provides an interesting tool to further understand how galanin receptors modulate synaptic transmission. Further studies with NAX-5055 will help illuminate the physiological role of galanin in controlling seizure activity.

Introduction

Galanin is a 29 (30 in humans) amino acid neuropeptide that displays a broad expression pattern within the central and peripheral nervous systems (Tatemoto et al., 1983; Lang et al., 2007). Interest in elucidating a detailed mechanism of action for galanin stems from its known modulatory effects on a number of neurological phenomena including neuronal excitability, neuroendocrine regulation, neuronal development, and cell survival (Hobson et al., 2008; Lerner et al., 2008; Mechenthaler, 2008). These physiological actions of galanin have been demonstrated to serve important roles in the endogenous response of the central nervous system to neurological disorders such as epilepsy, Alzheimer's disease, neuropathic pain, and mood disorders (Lundström et al., 2005; Wiesenfeld-Hallin et al., 2005; Crawley, 2008; Lerner et al., 2008; Rotzinger et al., 2010).

Galanin's ability to modulate neuronal excitability may be particularly relevant within the hippocampal formation, a region known to be prone to seizure generation. Within the hippocampus, galanin is expressed in efferent cholinergic and noradrenergic

fibers originating in the medial septum/diagonal band and locus coeruleus, respectively (Melander et al., 1986; Gabriel et al., 1995; Xu et al., 1998). There are three known galanin receptors (GalR1, 2, and 3) that all belong to the G-protein coupled receptor family (Habert-Ortoli et al., 1994; Parker et al., 1995; Smith et al., 1997) and [^{125}I]-galanin binding and *in situ* hybridization studies have demonstrated moderate to high expression levels of both GalR1 and GalR2 receptors within the hippocampus. GalR1 receptors are expressed throughout the corna ammonis (CA) fields of the ventral hippocampus, while GalR2 receptors are highly expressed in the dentate gyrus and display more moderate expression in the CA fields (Burgevin et al., 1995; O'Donnell et al., 1999; Lu et al., 2005b). Thus the neuroanatomical organization of the galanin system is well positioned to modulate hippocampal activity. Unfortunately, the lack of specific commercially available antibodies to the galanin receptors has made it difficult to confirm cell type specific protein expression (Lu and Bartfai, 2009).

Within the CNS, galanin is coexpressed with a number of classical neurotransmitters including acetylcholine, catecholamines, and serotonin and is thought to inhibit the release of these neurotransmitters during periods of high neuronal activity (Melander et al., 1986; Fisone et al., 1987; Tallent, 2008). These effects on neurotransmitter release are believed to occur though galanin receptor-mediated modulation of ATP-dependent K^+ channels, G-protein inwardly rectifying K^+ (GIRK) channels or voltage-gated Ca^{2+} channels (Palazzi et al., 1991; Zini et al., 1993; Jacoby et al., 2002; Kerekes et al., 2003; Endoh et al., 2008; Anselmi et al., 2009). Within the hippocampal formation, activation of galanin receptors by the active galanin fragment Gal(1-16) resulted in a 50% reduction of both glutamate and aspartate release following a

depolarizing stimulation (Zini et al., 1993). Further, in similar experiments performed with mice overexpressing galanin selectively in noradrenergic fibers, glutamate release was also significantly reduced following hippocampal slice depolarization. In contrast, in hippocampal slices acquired from galanin knockout mice, glutamate release is nearly doubled following the same depolarizing stimulation (Mazarati et al., 2000).

The robust inhibitory effects of galanin on the release of glutamate release in the hippocampus have lead to more rigorous electrophysiological analysis of this activity. At the mossy fiber-CA3 synapse in hippocampal slices, galanin overexpression resulted in a lowered frequency facilitation of the excitatory postsynaptic field potential (fEPSP), a form of short-term plasticity. This change in frequency facilitation was inhibited by the non-selective galanin antagonist M35 in the same galanin-overexpressing mice, confirming that this effect was mediated by galanin receptors (Kokaia et al., 2001). Galanin has also been shown to impair CA1/CA3 long term potentiation (LTP) in acute hippocampal slices, as well as dentate gyrus LTP both in hippocampal slice and *in vivo* (Coumis and Davies, 2002; Badie-Mahdavi et al., 2005b; Kinney et al., 2009). Taken together these data strongly support a role for galanin in the regulation of hippocampal excitatory synaptic transmission.

Within the hippocampal formation, galanin expression is highly plastic in response to experimentally induced seizure activity. Following status epilepticus induced by either Li-pilocarpine or perforant path stimulation, galanin immunoreactivity is drastically reduced in efferent fibers within 30 min. However, within 24 hr after status epilepticus or hippocampal kindling there is *de novo* expression of galanin mRNA in the dentate gyrus (Mazarati et al., 1998; Kokaia et al., 2001). In animals overexpressing

galanin, high levels of expression are observed in the dentate granuale cell layer, and this pattern of expression is enhanced and extended into the mossy fiber projection following hippocampal kindling (Kokaia et al., 2001; Lin et al., 2003; Kuteeva et al., 2005).

It remains unclear how the two galanin receptors mediate the effects of galanin on hippocampal excitatory synaptic transmission or seizure activity. Similar to many neuropeptide receptors, the GalR1 receptor selectively couples to the G_i-type G-protein, reducing cAMP through inhibition of adenylyl cyclase, opening GIRK channels, and stimulating mitogen-activated protein (MAP) kinase activity (Branchek et al., 2000; Lang et al., 2007). These intracellular functions suggest that the GalR1 receptor is well suited to regulate neuronal excitability. In contrast, the GalR2 receptor displays a more complicated signaling cascade, and it is capable of interacting with numerous G-proteins. The most commonly observed pathway involves the activation of phospholipase C (PLC), which results in the release of Ca^{2+} from intracellular stores and opening Ca^{2+}-dependent chloride channels that are potentially mediated by the $G_{q/11}$-type G-protein (Fathi et al., 1998; Wang et al., 1998). However, GalR2 has also been shown to couple to $G_{i/o}$ and $G_{12/13}$-type G-proteins in different immortalized cell lines (Lang et al., 2007). It is thus possible that activation of GalR2 receptors could result in cellular excitation or inhibition depending on which G-protein it is coupled to.

Previous attempts to generate systemically active, receptor-selective, galanin agonists have produced two compounds: galmic and galnon (Saar et al., 2002; Bartfai et al., 2004). However, both compounds were shown to interact with non-galanin receptors in the micromolar (μM) range, thereby limiting their use as pharmacological tools (Lu et al., 2005a). To address this issue, Bulaj and colleagues have successfully designed a

series of truncated galanin analogs in which nonessential amino acid residues are replaced by cationic and/or lipoamino acid residues (Bulaj et al., 2008). One analog from this group, NAX-5055, was demonstrated to possess a 15-fold preference for GalR1 over GalR2 with nanomolar (nM) affinity (Bulaj et al., 2008; White et al., 2009). NAX-5055 was found to be effective against 6 Hz psychomotor seizures; thus demonstrating that a GalR1-preferring galanin agonist can exert an antiseizure effect (Bulaj et al., 2008).

In this study, we hypothesized that the GalR1-preferring galanin analog NAX-5055 would reduce hippocampal excitability by modulating glutamatergic synaptic transmission. Furthermore, we hypothesized that this modulation would result from a presynaptic mechanism of action, consistent with the lowering of frequency facilitation observed in the presence of overexpressed galanin. To directly test this hypothesis, we recorded miniature excitatory postsynaptic currents (mEPSCs) from CA3 pyramidal neurons in the presence of NAX-5055 in mouse organotypic hippocampal slice cultures (OHSC). Analysis of the inter-event interval and amplitude of mEPSC events in CA3 pyramidal neurons allowed us to delineate the potential pre- and/or postsynaptic activity of NAX-5055.

Methods

Animals

Timed pregnant female C57BL/6J mice arrived in our animal facility at embryonic day 16 (Charles River, Kingston, WA). Mouse pups were born between 4-5 days after arrival. Animals were housed in a temperature-, humidity-, and light-controlled (12 h light:dark cycle) facility. Two dams were housed per cage and provided

free access to food (LabDiet) and water. All experimental procedures were performed in accordance with the guidelines established by the National Institutes of Health and received approval from the University of Utah's Animal Care and Use Committee. Dams and pups not used for slice culture preparation were humanely sacrificed by CO_2 asphyxiation.

Hippocampal Slice Preparation

Slice cultures were prepared using a modification of the the method originally described by Stoppini et al. (1991) (Alex et al., 2011). On postnatal day 5 (P5), C57BL/6J mouse pups were anesthetized with pentobarbital (25 mg/mL) and rapidly decapitated. Brains were extracted and the cerebellum was removed. Hemispheres were cut into 350 μm transverse sections using a McIlwain tissue chopper (Stoelting Co., Wood Dale, IL) and placed in chilled Gey's Balanced Salt Solution (GBSS, Sigma-Aldrich, St. Louis, MO) supplemented with 6.5 mg/mL glucose. Sections were separated under a dissection microscope and excess cortex removed to obtain intact hippocampal slices with the entorhinal cortex attached. Hippocampal sections were transferred to tissue culture membrane inserts (Millipore) in a 6-well tissue culture dish containing medium consisting of 50% minimum essential medium with Glutamax and HEPES, 25% Hanks balanced salt solution, 25% heat inactivated horse serum (all from Sigma-Aldrich, St. Louis, MO), and 6.5 mg/mL glucose. Medium was changed three times per week. Cultures were maintained at 37°C with 5% CO_2 balanced humidified air in a standard water-jacketed incubator (Thermo Scientific, Marietta, OH).

Electrophysiological Recording

On the day of recording, a membrane insert with 3-4 hippocampal cultures was removed from the 6-well culture plate and placed in a sterile petri dish that contained external recording solution containing (in mM): 150 NaCl, 3 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 10 HEPES, 10 glucose at pH 7.30 and osmolarity of 305 mOsm. External solution was stored in the incubator at 37°C. Slices were removed from petri dishes by cutting a portion of the membrane insert surrounding a single slice with a sterile scalpel and immediately placed into a perfusion recording chamber. The remaining slices on each insert were returned to the incubator for later use. Once in the recording chamber, slices were submerged and perfused at 2 mL/min with external recording solution. All recordings were performed at room temperature. To isolate miniature excitatory postsynaptic currents (mEPSCs), tetrodotoxin (TTX, 1 μM) and picrotoxin (PTX, 50 μM) were added to the external recording solution immediately before use. Whole cell recording pipettes (3-5 MΩ) were pulled on a PIP5 pipette puller (HEKA Instruments Inc., Bellmore, NY) and filled with internal recording solution containing (in mM): 140 $CsMeSO_4$, 20 HEPES, 5 EGTA (CsOH), 0.5 $CaCl_2$, 10 Glucose, 2 Na_2ATP, 0.5 NaGTP, 5 TEA-Cl at pH 7.30 and osmolarity of 300 mOsm. Biocytin (2 mg/mL) was added to intracellular recording solutions immediately before use to allow for visualization of the location and morphology of patch-clamped neurons.

Whole cell recordings were obtained using the visualized patch technique. The pyramidal cell layer of CA3 was visualized with a 40x water immersion objective (NA 0.8, Carl Zeiss, Thornwood, NY) using infrared differential interference contrast (IR-

DIC) microscopy on an upright Axioskop2 microscope (Carl Zeiss, Thornwood, NY). Voltage-clamp recordings were obtained in the whole-cell patch configuration using an Axopatch 200B amplifier and the CLAMPEX software package interfaced to a Digidata 1322A data acquisition board (Axon Instruments, Union City, CA). Recordings were acquired at 10 kHz for offline analysis using Clampfit 9 and Mini Analysis. Neurons were voltage clamped to -70 mV for the duration of the experiment. Membrane (R_m) and access (R_a) resistance were measured by using a 5-mV voltage step at the end of every 30-second sweep for the duration of the recording. Fluctuations greater then 20% of baseline holding current, R_m, or R_a across the duration of the experiment resulted in cells being excluded from further analysis. Only one cell was recorded per slice. The results from a total of 6 cells were included in this study.

After obtaining a gigaohm seal and entering the whole-cell configuration, voltage-clamped neurons were allowed to stabilize for approximately 10 min. Based on the stability of the holding current, R_a, and R_m, experiments with NAX-5055 were initiated or cells were abandoned. mEPSCs were recorded continuously for 35 min; 10 min of baseline in the presence of TTX and PTX, followed by a 15 min perfusion of NAX-5055, followed by a10 min drug washout period.

Immunohistochemistry and Imaging

At the conclusion of the recording protocol, slice cultures were immediately transferred to culture wells containing 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 24 hr at 4°C for fixation, and subsequently stored in 1x PBS until staining. For slice processing, slices were rinsed twice in 1x PBS and blocked with 4.5%

normal goat serum in 0.3% PBT (1x PBS + 0.3% Triton-X 100) for 4 hr. Slices were then incubated overnight with streptavidin conjugated to Cy3 (1:500) and NeuN conjugated to Alexa 488 (1:500) in 0.1% PBT and 4.5% normal goat serum at room temperature. On the following day, slices were rinsed in 1x PBS containing 4′,-6-diamidino-2-phenylindole (DAPI, 0.1 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min, followed by the final wash steps in 1x PBS. Slices were then mounted and coverslipped in Prolong gold anti-fade reagent (Invitrogen, Carlsbad, CA).

Slides were imaged to confirm cell morphology and location under epifluoresence conditions using an upright Axio Imager.A1 microscope (Carl Zeiss, Thornwood, NY) and mercury arc lamp.

Analysis

All mEPSCs were detected and analyzed using Mini Analysis software (Synaptosoft, Fort Lee, NJ). Prior to detection traces were subject to a low-pass Elliptic filter at 700 Hz. The detection threshold for mEPSCs was set to 10 pA, to limit inclusion of false positive events.

In order to normalize raw data, a percent control value was calculated for inter-event interval and amplitude of mEPSCs, as well as holding current, R_m, and R_a. Percent control was calculated by taking an average of the values recorded during the final 2 minutes of baseline. Each raw value was then divided by the generated average and multiplied by 100 resulting in an individual percent control value. Time course data is presented as mean ± standard error of the mean (SEM).

To quantify differences in inter-event interval and amplitude of mEPSCs, recorded values during the final 5 minutes of each recording period (baseline, NAX-5055, and washout) for each cell were used and are presented as mean ± SEM. A paired t-test was used to determine significance between recording periods. Significance was set at $p < 0.05$. Analysis was completed with Microsoft EXCEL and GraphPad Prism software (La Jolla, CA).

Results

Mouse Organotypic Hippocampal Slice Cultures

To determine if our culture methods would alter the characteristic structural lamination of the hippocampus, we stained mouse organotypic hippocampal slice cultures (OHSC) with the neuronal marker NeuN after at least 7 days *in vitro* (DIV). Staining patterns demonstrate that the classic lamination of pyramidal cell layers is maintained in our slice cultures, with the dentate gyrus and both CA1 and CA3 clearly visible and easily distinguished (Figure 2.1A). All recorded cells were filled with biocytin during recording and then fixed and processed for immunofluorescence to determine cell type and confirm location. Figure 2.1B illustrates a biocytin-filled pyramidal neuron within the CA3 region of the OHSC that co-localizes with the neuronal marker NeuN. On occasion, astrocytes were patch clamped and were excluded from further analysis.

Inter-event Interval and Amplitude of mEPSCs

It has been suggested that the anticonvulsant properties of the neuropeptide galanin result from galanin's inhibition of glutamate release within the hippocampus

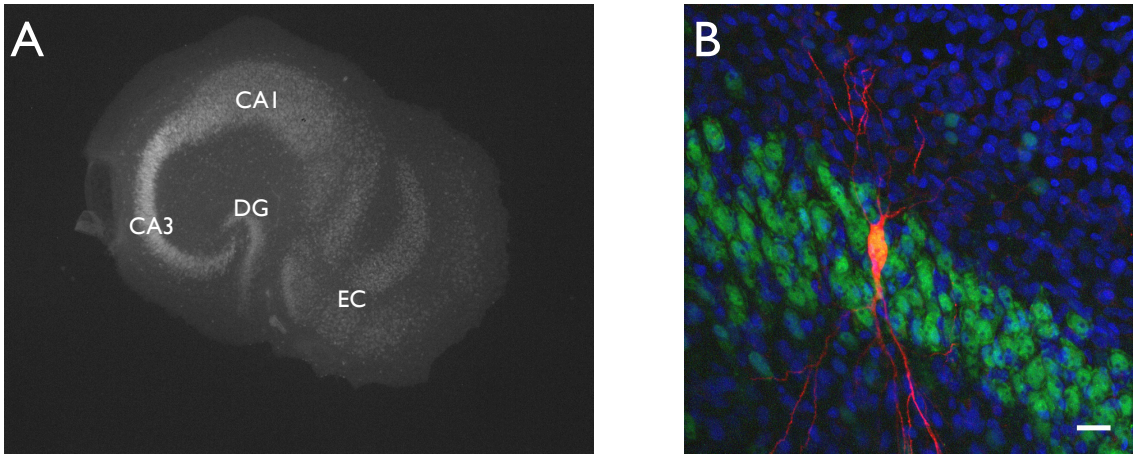


Figure 2.1. Images of a typical organotypic hippocampal slice in culture and biocytin-filled CA3 pyramidal neuron.

A) NeuN stain of a typical 7 DIV mouse organotypic hippocampal slice culture that was used in recordings. Hippocampi retain their laminar structure with the dentate gyrus (DG) and pyramidal cell layers CA3 & CA1 remain intact. Slices were cultured with the entorhinal cortex (EC) attached.

B) Staining for biocytin following whole-cell recordings. Biocytin (red) perfuses throughout recorded CA3 pyramidal neuron allowing for visualization of neuronal morphology and location within the slice. Neuronal identity is confirmed by co-labeling with the neuronal marker NeuN (green). DAPI is shown in blue. Scale bar, 20 μm.

Figure 2.2. NAX-5055 increased the mEPSC inter-event interval in mouse OHSC.

A) Recording in the presence of TTX (1 μM) and PTX (50 μM) blocked all action potential- and GABA-mediated events allowing for resolution of the mEPSCs. These events were considered the baseline for each cell recorded (n = 6 cells)

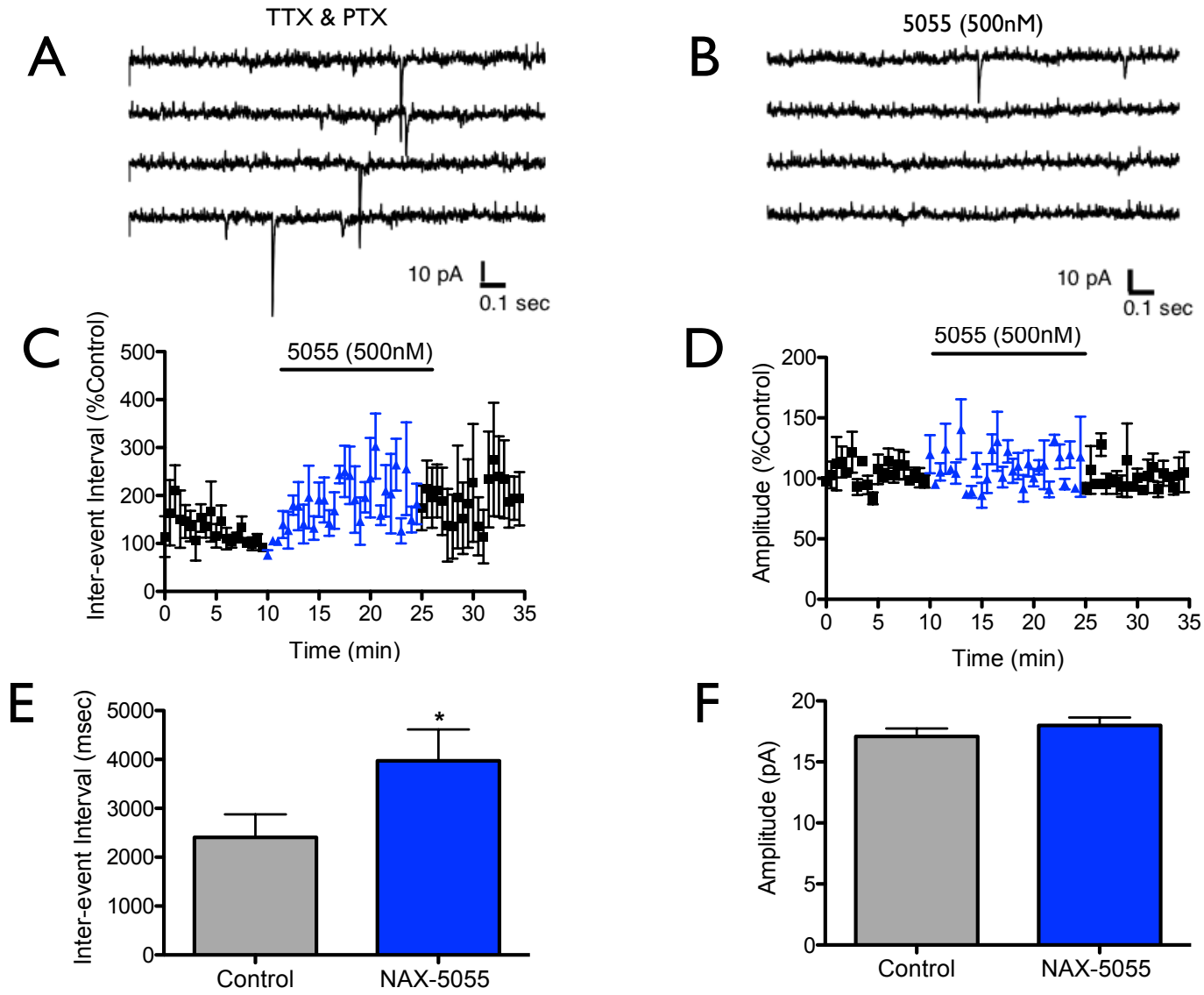
B) Representative traces of mEPSCs recorded during the 15-min perfusion of NAX-5055 (500 nM).

C) Inter-event interval expressed as percent control begins to increase following perfusion of NAX-5055 (blue) and continues to increase for the duration of the 15-min exposure. Values do not return to baseline during a 10-min washout period.

D) The amplitude of mEPSCs expressed as percent control remains relatively stable for the duration of the recordings.

E) There is a significant increase in the inter-event interval during the last five minutes of recording in the presence of NAX-5055 (paired t-test, $p < 0.05$).

F) There is no significant difference in the amplitude of mEPSCs during the last five minutes of recording in the presence of NAX-5055 (paired t-test, $p > 0.05$


A
TTX & PTX
10 pA
0.1 sec
B
5055 (500nM)
10 pA
0.1 sec
C
5055 (500nM)
Inter-event Interval (%Control)
Time (min)
D
5055 (500nM)
Amplitude (%Control)
Time (min)
E
Inter-event Interval (msec)
Control
NAX-5055
F
Amplitude (pA)
Control
NAX-5055

(Zini et al., 1993; Mazarati et al., 2000). To elucidate whether the anticonvulsant efficacy of the GalR1-preferring analog NAX-5055 exhibits a similar mechanism of action, we measured the effect of NAX-5055 on mEPSCs in CA3 pyramidal neurons. Figures 2.2A and 2.2B show representative traces of recorded mEPSCs under baseline conditions and after NAX-5055 (500 nM) exposure, respectively. NAX-5055 (500 nM) produced a gradual increase in the inter-event interval across the duration of the 15 min drug-wash period. However, the 10 min washout period was insufficient to reverse this effect (Figure 2.2C). There was little variability in the amplitude of mEPSCs throughout the duration of the recordings (Figure 2.2D). Quantification of the last 5 min of each recording period (baseline, NAX-5055, and washout) demonstrated a significant increase in the inter-event interval in the presence of NAX-5055 when compared to baseline levels (Figure 2.2E, $p < 0.05$, $n = 6$ cells). In contrast, there was no change in amplitude of mEPSCs (Figure 2.2F, $p > 0.05$, $n = 6$ cells). There was no significant difference between NAX-5055 and the washout period for either inter-event interval or amplitude (data not shown).

Discussion

In this study we have shown that the GalR1-preferring galanin analog, NAX-5055 increases the inter-event interval of mEPSCs without affecting the amplitude of these events in CA3 pyramidal neurons in mouse OHSC. Together, these data suggest that the site of activity for NAX-5055 is largely presynaptic. To our knowledge, this is the first demonstration of a synthetic galanin analog found to affect the probability of presynaptic glutamate release within the hippocampal formation. Further, these observations suggest

that one mechanism of action for the anticonvulsant activity of NAX-5055 is the reduction of neuronal excitability via inhibition of presynaptic glutamate release onto CA3 pyramidal neurons.

It has been well documented that organotypic slice cultures retain the anatomical and physiological properties that are observed in more classically used acute hippocampal brain slices. (Zimmer and Gähwiler, 1984; 1987; Gähwiler et al., 1997). Thus, it seems unlikely that differences in the expression and localization of galanin receptors in the OHSC account for the affects we have observed in the present study. In fact, preliminary evidence in our lab has suggested that NAX-5055 also increases inter-event interval of mEPSCs without affecting amplitude in CA3 pyramidal neurons in acute hippocampal brain slices from C57BL/6J mice. These data are consistent with what we have reported here using mouse OHSC.

Utilization of mouse OHSC allows for future study of the physiological effects associated with repeated agonist exposure of galanin receptors expressed on pyramidal neurons. This is particularly interesting given that both the GalR1 and GalR2 receptors have been shown to internalize following agonist exposure in immortalized cell lines expressing either receptor type (Wang et al., 1998; Xia et al., 2005; 2008). Once internalized, GalR1 receptors are known to be trafficked to lysosomes and degraded (Xia et al., 2008). However, the regulation and trafficking patterns of galanin receptors have yet to be verified in neurons. In addition, repeated systemic administration of NAX-5055 results in a rapid decrease in anticonvulsant efficacy and the mechanism of this response remains unclear (see Chapter 3). Characterization of the physiological activity of NAX-

5055 in mouse OHSC will allow us to further address how galanin receptors are regulated in functional neurons.

The observed reduction of glutamate release in the hippocampus by NAX-5055 is consistent with previous work demonstrating that exogenously applied or overexpressed galanin can reduce glutamate release in acute hippocampal slices (Zini et al., 1993; Mazarati et al., 2000). Short-term plasticity studies of the mossy fiber-CA3 synapse have been utilized to assess the potential presynaptic mechanism for the galanin-induced reduction in glutamate release (Zucker and Regehr, 2002). Under basal conditions the mossy fiber-CA3 synapse displays short-term plasticity in the form of paired-pulse facilitation (PPF) and frequency facilitation; an action likely due to a low probability of vesicular release (Cremer et al., 1998; Lawrence et al., 2004; Nicoll and Schmitz, 2005). In transverse hippocampal slices from mice overexpressing galanin via the platelet derived growth factor B promoter, frequency facilitation of fEPSPs at the mossy fiber-CA3 synapse is lowered, suggesting galanin can act presynaptically (Kokaia et al., 2001). However, no change in PPF or frequency facilitation of fEPSPs is observed in dorsal hippocampal slices when galanin is overexpressed using a recombinant adeno-associated viral vector (Kanter-Schlifke et al., 2007). This lack of effect may result from the low expression of galanin receptor mRNA in the dorsal hippocampus (Burgevin et al., 1995). Although changes in short-term plasticity suggest a presynpatic mechanism for galanin, analysis of mEPSCs can not distinguish an increase or decrease in presynaptic glutamate release. Our data supports the hypothesis that galanin limits hippocampal excitability through presynaptic mechanisms and also expands this hypothesis by suggesting that galanin could interact with GalR1 receptors to reduce presynaptic glutamate release onto

CA3 pyramidal neurons. Further study is required in order to demonstrate that it is indeed the GalR1 receptor that mediates the inhibitory effect in NAX-5055 in CA3.

In the dentate gyrus, galanin and the nonpeptide agonist galmic inhibit PPF of fEPSPs following a 500-1000 ms inter-stimulus interval (Badie-Mahdavi et al., 2005a; 2005b). The long inter-stimulus intervals appear critical for this effect, as no effect of galanin on PPF was observed in other studies utilizing shorter inter-stimulus intervals (Mazarati et al., 2000; Kinney et al., 2009). Isolation of the GalR2 receptor using the galanin fragment Gal(2-11) produced no effect on PPF at any inter-stimulus interval (Badie-Mahdavi et al., 2005b). In the GalR1 knockout mouse, neither galanin nor Gal(2-11) had any effect on PPF, suggesting that the delayed reduction in PPF seen with galanin and galmic is GalR1 dependent (Badie-Mahdavi et al., 2005b). Thus, similar to what we report in CA3, galanin may modulate presynaptic release through a GalR1-dependent mechanism within the dentate gyrus. Additional studies with NAX-5055 in the dentate gyrus and other regions of the hippocampus will further clarify the role of GalR1 in modulating hippocampal excitability.

Although our work has focused on the anti-seizure effects of NAX-5055, galanin has been also been well documented to negatively impact cognition. Indeed, central administration or overexpression of galanin impairs performance in a number of learning and memory tasks (Crawley, 2008). Consistent with these behavioral effects, galanin and Gal(2-11) have been shown to attenuate long term potentiation (LTP) in both the dentate gyrus and CA1 induced by either high frequency stimulation or theta-burst stimulation (Coumis and Davies, 2002; Badie-Mahdavi et al., 2005b; Kinney et al., 2009). It has been suggested that the inhibitory effects of galanin working through the GalR2 receptor

on LTP are postsynaptic, based on the inability of Gal(2-11) to inhibit PPF (Badie-Mahdavi et al., 2005b). However, it remains unclear what role the GalR1 receptor plays in the modulatory affects of galanin on LTP. The results of the studies reported here suggest that GalR1 receptors could modulate neuronal excitability through a presynaptic mechanism. It is interesting to speculate that galanin may affect LTP through both pre- and post-synaptic mechanisms, mediated by the two different receptor subtypes. Studies utilizing the receptor preferring agonists NAX-5055 and Gal(2-11) will allow for more rigorous testing of this hypothesis.

Although NAX-5055 provides us a novel tool to study galanin receptors, it is curious that the inter-event interval of mEPSCs dose not return to pre-NAX-5055 levels following the washout period. In these studies, a 10 min washout period was always used, however in preliminary studies washout periods of up to 40 min were insufficient for inter-event interval values to return to baseline. The inability to washout the effects if NAX-5055 could result from activating G-protein coupled receptor signaling cascades, which can modulate cellular activity for minutes to hours after agonist washout. In addition, the lack of washout may result from the chemical modifications that were added to NAX-5055 to increase its metabolic stability *in vivo*. In particular, the addition of a lysine-palmitoyl tail that increases the lipophilicity of NAX-5055 (Bulaj et al., 2008), may make the peptide difficult to completely washout of the slice preparation. As a result it may be difficult to completely remove NAX-5055 once it has been added to the slice. Additional studies using the scrambled NAX-5055 isomer 805-1, which lacks binding affinity for galanin receptors will help address this issue.

In conclusion we have shown that NAX-5055 reduces presynaptic glutamate release onto CA3 pyramidal neurons. This action may serve as one mechanism to explain the anti-seizure activity of this peptide. To our knowledge, this is the first demonstration of a GalR1-preferring galanin analog directly affecting the probability of glutamate release in the hippocampus. These observations provide the groundwork to further study the role of the GalR1 receptor in the regulation of neuronal excitability within the hippocampus.

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CHAPTER 3

DIFFERENTIAL ANTISEIZURE EFFECTS OF THE GALR1 PREFERRING AGONIST NAX-5055 FOLLOWING ACUTE AND REPEATED ADMINISTRATION

Abstract

The neuropeptide galanin is widely expressed in the central nervous system and plays an important role in inhibiting seizure activity. There are three cloned galanin G-protein coupled receptors (GalR1-3). GalR1 and GalR2 receptors are expressed in brain regions prone to hyperexcitability. The role of each receptor subtype in the inhibition of seizure activity remains unclear. To address this issue we have developed the GalR1-preferring galanin analog NAX-5055 that displays potent anti-seizure activity in a variety of epilepsy and seizure models. The goal of the present study was to acess the disease-modifying potential of NAX-5055 in the corneal kindled mouse model of partial epilepsy. When administered by intraperitoneal (i.p.) injection one hour prior to each kindling stimulation, NAX-5055 did not exert any effect on the rate of kindling acquisition. We next evaluated the antiseizure efficacy of NAX-5055 following acute or repeated dosing in the mouse 6 Hz seizure test, a model of partial seizure. A single acute dose of NAX-5055 was effective in the 6 Hz test. In contrast, the efficacy of NAX-5055 was markedly reduced when it was administered i.p. once daily for three consecutive days prior to the 6 Hz challenge. As a result, the focus of this study shifted to

understanding potential mechanism(s) that could underlie the reduced efficacy of NAX-5055 following repeated administration. To test the role of central galanin receptors, dose response studies were conducted. We compared mice treated with an acute dose or repeated (once daily (i.p.) for three consecutive days followed by a final challenge) doses of NAX-5055. The acute or final dose of NAX-5055 was administered i.p. or intracerebroventricular (i.c.v.) prior to the 6 Hz test. A significant rightward shift of the dose response curve was noted in the group that received repeated i.p. doses of NAX-5055. In contrast, when the last dose of NAX-5055 was administered i.c.v., no significant difference in the dose-response curves between groups was observed. These results suggest that central galanin receptors can mediate anti-seizure activity if NAX-5055 can gain access to them. In a subsequent study designed to evaluate the role of efflux transport, it was found that NAX-5055 does not serve as a substrate for the efflux transporter p-glycoprotein (P-gp). These results suggest that the reduction in anti-seizure activity following repeated NAX-5055 administration is not dependent on modified central galanin receptor availability or increased efflux transport by P-gp. However, the reduction in potency of following repeated NAX-5055 adminstration may explain the failure of NAX-5055 to affect kindling acquisition rate. Additional investigations are required before a complete understanding of this phenomenon can be determined.

Introduction

It is estimated that there are 50 million epilepsy patients worldwide, with nearly 150,000 new cases diagnosed annually in the United States alone (Hirtz et al., 2007; Institute of Medicine, 2012). Approximately 30% of epilepsy patients are considered

drug-resistant. These patients display inadequate seizure control following treatment with at least two different antiepileptic drugs in either mono- or poly-therapy (Kwan and Brodie, 2003; Cascino, 2008; Kwan et al., 2010a). Neuropeptides, including galanin and neuropeptide Y, display increased expression following experimentally induced seizures (Wilson et al., 2005). In addition, neuropeptides display antiseizure activity in a variety of epilepsy animal models (Baraban, 2004; Lang et al., 2007). Thus, neuropeptides and their cognate receptors provide novel, pathologically relevant, targets for therapeutic development (Robertson et al., 2011; Weinberg and McCown, 2011).

Galanin is a 29-amino acid (30 in humans) endogenous neuropeptide expressed throughout the central nervous system; including limbic brain structures known to be involved in ictogenesis (Lang et al., 2007; Lerner et al., 2008). Within the hippocampus, galanin is expressed in cholinergic projections from the medial septum and noradrenergic projections originating from the locus coeruleus (Consolo et al., 1994; Mazarati et al., 1998; Xu et al., 1998). In animal models of status epilepticus, galanin immunoreactivity within these projections is markedly reduced, likely due to increased release and exhaustion of galanin stores (Mazarati et al., 1998; Lerner et al., 2008). However, *de novo* neuronal immunoreactivity in the hilar region and increased mRNA expression of galanin throughout the hippocampus is also observed following status epilepticus (Mazarati et al., 1998; Wilson et al., 2005). These observations suggest endogenous galanin levels display robust plasticity in response to seizure activity.

Investigation of the antiseizure properties of galanin has relied heavily on experimentally enhancing central galanin expression levels. Direct galanin injection into the hilus or overexpression using transgenic mice leads to increased resistance to the

induction of status epilepticus and reduced pentylenetetrazol-induced seizure severity (Mazarati et al., 1992; 1998; 2000). Overexpression of galanin also increases after-discharge threshold in kindled mice and delays the rate of kindling acquisition (Kokaia et al., 2001; Kanter-Schlifke et al., 2007). One potential mechanism for the anti-seizure activity of galanin is inhibition of stimulated glutamate release within the hippocampus (Zini et al., 1993; Mazarati et al., 1998). Together these studies suggest that galanin, at experimentally increased concentrations, can effectively modulate neuronal excitability and seizure activity.

There are three known galanin receptors (GalR1, 2, and 3) belonging to the G-protein coupled receptor family (Habert-Ortoli et al., 1994; Parker et al., 1995; Smith et al., 1997). All three galanin receptors are expressed at varying levels throughout the brain (for review see (Branchek et al., 2000)). [^{125}I] galanin binding and *in situ* hybridization studies are consistent in the demonstration of moderate to high expression patterns for both GalR1 and GalR2 receptors within the ventral hippocampus (Burgevin et al., 1995). High levels of GalR2 receptor expression are also observed in the dentate gyrus (O'Donnell et al., 1999). GalR3 receptors are expressed at low levels in the brain, with expression in discrete regions including hypothalamus and pituitary (Smith et al., 1997). However, the lack of specific galanin receptor antibodies has made it difficult to confirm region and cell-type specific protein levels within the brain (Lu and Bartfai, 2009).

Similar to many neuropeptide receptors, the GalR1 receptor selectively couples to the G_i-type G-protein (Branchek et al., 2000). Receptor activation inhibits forskolin-stimulated cAMP production (Habert-Ortoli et al., 1994), opens G-protein regulated

inwardly rectifying K^+ (GIRK) channels (Smith et al., 1998), inhibits voltage dependent Ca^{2+} influx (Endoh et al., 2008; Anselmi et al., 2009), and stimulates mitogen-activated protein kinase activity (Wang et al., 1998b). The selective coupling of GalR1 to an inhibitory G-protein with downstream effects that can induce cell hyperpolarization make this receptor an interesting target for antiseizure drug development.

In contrast, the GalR2 receptor displays a more complicated signaling cascade capable of interacting with numerous G-proteins. The most commonly observed pathway involves a $G_{q/11}$-type G-protein mediated activation of phospholipase C (PLC) resulting in release of Ca^{2+} from intracellular stores and opening Ca^{2+}-dependent chloride channels (Fathi et al., 1998; Wang et al., 1998a). Interestingly, GalR2 has also been shown to couple to $G_{i/o}$ and $G_{12/13}$-type G-proteins in immortalized cell lines (Lang et al., 2007). It is thus possible that activation of GalR2 receptors could result in neuronal excitation or inhibition depending on specific G-protein coupling at the time of receptor activation.

It is currently unclear what role each galanin receptor subtype plays in mediating the anti-seizure properties of galanin. Studies investigating transgenic mice have demonstrated that knock-out of the GalR1 receptor or knockdown of the GalR2 receptor increases seizure severity and cellular damage in status epilepticus animal models (Mazarati et al., 2004a; 2004b; Schauwecker, 2010). In addition, spontaneous seizures have been observed in the GalR1 knockout mouse, further implicating this receptor in the regulation of neuronal excitability (McColl et al., 2006). Despite these advances, there is still a lack of receptor-selective, systemically available agonists to aid in evaluating the role of each receptor subtype in mediating the antiseizure activity of galanin.

Previous attempts to generate systemically active galanin agonists produced two compounds: galmic and galnon (Saar et al., 2002; Bartfai et al., 2004). However, both compounds were found to interact with non-galanin receptors in the micromolar (μM) range; thereby limiting their use as pharmacological tools (Lu et al., 2005). To address this issue Bulaj and colleagues successfully designed a series of truncated galanin analogs in which nonessential amino acid residues were replaced by cationic and/or lipoamino acid residues (Bulaj et al., 2008). One analog from this group, NAX-5055, displays nanomolar affinity for both galanin receptors and a 15-fold preference for GalR1 over GalR2 (Bulaj et al., 2008; White et al., 2009). Furthermore, NAX-5055 possesses a broad-spectrum anti-seizure profile in a variety of animal seizure and epilepsy models following systemic administration. This profile provides important proof of concept that a GalR1-preferring galanin agonist can inhibit seizure activity *in vivo* (Bulaj et al., 2008).

In this study we sought to further characterize the anti-seizure activity of NAX-5055. Previous studies have demonstrated that kindling acquisition is delayed in mice and rats overexpressing galanin in the brain (Kokaia et al., 2001; Kanter-Schlifke et al., 2007). In this study we evaluated the potential of NAX-5055 to modulate the rate of kindling acquisition in the corneal kindled mouse model of partial epilepsy. Given early results in the 6 Hz seizure model suggesting that the efficacy of NAX-5055 was reduced with repeated administration, we became interested in the mechanism(s) underlying this effect. NAX-5055 preferentially interacts with GalR1 receptors, which have been shown to be internalized and degraded following acute agonist exposure in immortalized cell lines (Wang et al., 1998a; Xia et al., 2008). Thus, we evaluated the effects of repeated activation of galanin receptors on the anti-seizure potency of NAX-5055 in an effort to

establish whether receptor availability contributes to the observed decrease in efficacy. This study offers the first report describing the effects of repeated treatment with a GalR1-preferring galanin analog in animal seizure and epilepsy models.

Methods

Animals

Adult male CF-1 mice weighing at least 18g (Charles River, Kingston, WA) where used for all experiments described in this study. Animals were housed in a temperature-, humidity-, and light-controlled (12h light:dark cycle) facility. Mice were group housed and allowed access to food and water *ad libitum*. At the conclusion of all experiments animals were sacrificed immediately by CO_2 asphyxiation. All experimental procedures were performed in accordance with the guidelines established by the National Institutes of Health and approved by the University of Utah's Animal Care and Use Committee.

Seizure and Epilepsy Models

Corneal Kindling Acquisition

CF-1 mice were kindled according to the methods originally defined by Matagne and Klitgaard (Matagne and Klitgaard, 1998). Briefly, mice were stimulated twice daily with a sub-convulsive corneal stimulation of 3 mA (60 Hz) for a 3-second duration. Daily stimulations were delivered with at least 4 hrs between stimulations. Prior to each stimulation, a drop of 0.9% saline containing 0.5% tetracaine hydrochloride (Sigma-Aldrich, St. Louis, MO) was applied to the cornea to ensure local anesthesia and good

electrical conductivity. The evoked behavioral seizures were classified according to the five-stage Racine scale (Racine, 1972). Stages 1 to 3 are considered partial seizures and stages 4 and 5 generalized seizures. Mice not displaying any behavioral seizure activity were given a score of 0. Mice were considered fully kindled after they had displayed five consecutive stage five seizures (see Figure 3.1). Stimulations were continued until vehicle-treated mice reached a fully kindled state. The corneal kindling model displays a behavioral phenotype and pharmacological profile that is consistent with human partial epilepsy (Rowley and White, 2010).

When testing NAX-5055 against fully kindled seizures mice were stimulated the day before testing to ensure the maintenance of the kindled state (n = 8 per treatment). To determine the effect of NAX-5055 on kindling acquisition, mice were administered NAX-5055 1 hr prior to each corneal stimulation for the duration of the kindling acquisition phase (i.e., two times daily) (NAX-5055-treated, n = 24; Vehicle-treated, n = 16). Kindling acquisition rate was determined by the number of stimulations required to reach each behavioral seizure stage and the fully kindled state.

6 Hz psychomotor seizure test

Partial psychomotor seizures were evoked using an acute corneal stimulation (6 Hz, 0.2 msec rectangular pulse width, 3-sec duration) with current generated by a Grass S48 stimulator (Grass Technologies, West Warwick, RI). Current intensity was set to 32 mA approximately 1.5 times the convulsive current necessary to generate a prototypical seizure in 97% of male CF-1 mice (Barton et al., 2001). Prior to each stimulation, a drop of 0.9% saline containing 0.5% tetracaine hydrochloride (Sigma-Aldrich, St. Louis, MO)

was applied to the cornea to ensure local anesthesia and good electrical conductivity. Behavioral seizures were characterized by rhythmic twitching of the vibrissae, head nodding, forelimb clonus, and/or rearing and falling (Barton et al., 2001). Mice not observed to display any of the above phenotypic features were scored as protected (i.e., 100% protection).

Peptide Design and Preparation

The galanin analog NAX-5055 was originally designed and synthesized at the University of Utah as previously described (Bulaj et al., 2008). NAX-5055 was later synthesized in bulk by NeoMPS (San Diego, CA). The inactive scrambled analog of NAX-5055, i.e. 805-1, in which Tyr^2 and Trp^9 were swapped, was also designed and synthesized at the University of Utah. Solutions were prepared by dissolving synthesized peptides in 0.9% saline containing 1% Tween 20 (Sigma-Aldrich, St. Louis, MO). Solutions were made fresh daily. Prior to each experiment the peptide concentration of each solution was confirmed by UV absorbance of tyrosine and tryptophan residues (Cary 50 Bio UV Spectrophotometer, Varian).

Experimental Design and Dosing Regimen

Mice were removed from the colony, weighed, treated, tested, and either returned to the colony (in their home cage) or sacrificed between the hours of 8:00 AM and 5:00 PM. In all studies mice were randomly selected from their home cage and placed into experimental groups with no intentional bias. Compounds were administered intraperitoneally (i.p.) in a volume of 0.01 mL/g body weight or intracerbroventricularly

(i.c.v.) in a volume of 5μL. Whether mice received peptide or vehicle was dependent on the randomly assigned experimental group (see below). Seizure responses in each behavioral model (i.e., corneal kindling or 6 Hz) were assessed at the time to peak effect of NAX-5055 based on the route of administration: 1 hr for i.p. injection and 15 min for i.c.v. injection. All i.c.v. injections were done by free hand injection using a Hamilton microsyringe (Reno, NV) with a clear plastic guard to ensure proper needle depth.

Acute and Repeated Administration of NAX-5055 (4 mg/kg)

In experiments that compared the effects of single or repeated doses of NAX-5055, mice were split into one of three groups as depicted in Figure 3.2A (n = 8 per group). Mice in Group 1 (vehicle) received vehicle for 4 days. Mice in Group 2 (acute NAX-5055) received vehicle for the first 3 days and then a single injection of NAX-5055 on the 4th day. Mice in Group 3 (repeated NAX-5055) received NAX-5055 for all 4 days. Injections administered on days 1-3 were always given systemically (i.p.). Injections given on day 4 were administered either i.p. or i.c.v. After the last injection on day 4, animals in all groups were subjected to the 6 Hz test at 32 mA.

Repeated Administration of NAX-5055 and 805-1

In experiments designed to compare the effect of repeated doses of NAX-5055 with 805-1, mice were split into one of four treatment groups as depicted in figure 3.3A (n = 8 per group). Mice in Group 1 (vehicle) received vehicle for all 4 days. Mice in Group 2 (acute NAX-5055) received vehicle for the first 3 days and a single injection of NAX-5055 on the 4th day. Mice assigned to Group 3 (repeated NAX-5055) were treated

with NAX-5055 for all 4 days. Mice in the final group; i.e., Group 4 (805-1) received 805-1 for the first 3 days and then a single injection of NAX-5055 on the 4th day. One hour after the last injection on day 4, animals in all groups were subjected to the 6 Hz test at 32 mA. All treatments were administered i.p..

NAX-5055 Dose Response and ED_{50} Determination

For the dose response studies, mice were split into one of two groups. Mice in the first group (acute NAX-5055) received vehicle for the first 3 days and then a single injection of NAX-5055 on the 4th day. Mice in the second group (repeated NAX-5055) received NAX-5055 for all 4 days. Injections administered on days 1-3 were always given systemically (i.p.). Injections given on day 4 were administered either i.p. or i.c.v. After the last injection on day 4, animals in all groups were subjected to the 6 Hz test (32 mA 1 hr after dosing). Results from dose-response studies are expressed as the median effective dose (ED_{50}) with corresponding 95% confidence intervals. To generate these values, mice (in groups of at least n = 8) were tested at different doses of NAX-5055, until at least two points were established between the limits of 0% and 100% protection from seizure activity. The number of mice tested at each dose and the percentage of those mice that displayed protection from seizure activity were entered into a log probit analysis program to generate ED_{50} and 95% confidence interval values. Nonoverlapping confidence intervals were considered to demonstrate a significant shift in the dose-response curves between different treatment groups.

Mouse p-glycoprotein Substrate ATPase Assay

To evaluate the potential interaction between NAX-5055 and the efflux transporter P-glycoprotein (P-gp), ATPase activity was measured using an *in vitro* colorimetric assay developed by BD Biosciences (Sarkadi et al., 1992; Drueckes et al., 1995). Substrate compounds for P-gp result in the cleavage of ATP to ADP and release of a free phosphate group that can be measured by using UV spectroscopy. Membranes containing P-gp expressed from mouse mdr1a or mdr1b cDNA using a baculovirus expression system were obtained from Genetest (Woburn, MA). Reactions were run on a 96-well plate with 60-µL reaction mixtures. Each well contained 40 µg membrane protein, NAX-5055, and 5 mM MgATP (20 µL of each) in a 50 mM Tris buffer containing 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, and 5 mM sodium azide. The concentration of NAX-5055 was varied between 3-300 nM. Identical reactions were run in tandem with 100 µM sodium orthovanadate. Orthovanadate inhibits P-gp by trapping MgATP in the nucleotide binding site. This control increases the sensitivity of the assay by allowing for identification and subtraction of any non-specific ATPase activity. Verapamil, a known substrate for P-gp, was also run on all plates as a positive control. Reaction mixtures were incubated for 20 min at 37 °C. The reaction was stopped by the addition of 30 µl 10% SDS and Antifoam A. An additional set of reactions were run in which MgATP was added post SDS. The results from this reaction represent t = 0 min. Incubation was followed with the addition of 200 µl of the colorimetric reagent (35 mM ammonium molybdate in 15 mM zinc acetate combined with 10% ascorbic acid (pH 5.0) prepared each day (in a ratio of 1:4)). The liberation of inorganic phosphate was detected

by its absorbance at 850 nm and quantified by comparing the absorbance of individual wells to a phosphate standard curve using liner regression analysis.

Statistical Analysis

Analysis of the corneal kindling acquisition rate was determined by averaging the number of stimulations required to reach each seizure stage during the acquisition process. A two-way ANOVA was used to determine any difference in seizure scores observed between vehicle- and NAX-5055-treated groups.

For all experiments utilizing the 6 Hz psychomotor seizure test the non-parametric Kruskal-Wallis test with a Dunn's posttest analysis was used in order to compare treatment groups. This statistical test was used due to the binary nature of 6 Hz test data (protected or unprotected). Results of the ATPase assay were analyzed by fitting dose response data with the Michaelis-Menten model of simple enzyme kinetics to yield V_{max} and K_m values. All differences between groups were considered to be significantly different when $p < 0.05$. All statistical analysis described above was completed using GraphPad Prism Software (La Jolla, CA).

Results

Acute Anti-seizure Profile of NAX-5055

The anti-seizure profile for the GalR1-preferring analog NAX-5055 was established previously using a battery of well-established animal seizure and epilepsy models. The results of this investigation are summarized in Table 3.1 (White et al., 2009). NAX-5055 displays potent antiseizure activity in models of partial seizures

Table 3.1

Anti-seizure efficacy of NAX-5055 in animal seizure and epilepsy models following systemic administration

Animal model	ED_{50} (mg/kg)	Protective Index[a]
6 Hz psychomotor seizure (mouse)		
22mA	0.7	30
32mA	0.8	26.3
44mA	2.9	7.2
Fring's Audiogenic (mouse)	3.2	6.5
Corneal Kindled (mouse)	0.65	30
Hippocampal Kindled (rat)	2.0	10.5
Maximal Electroshock (mouse)	>20.0	<1
s.c. PTZ (mouse)	25% protection at 20.0	<1

[a] TD_{50}/ED_{50}; TD_{50} = 21 mg/kg
Adapted from White et al., 2009

including the 6 Hz psychomotor seizure model (at 22, 32, and 44 mA current intensities), the corneal kindled mouse, and the hippocampal kindled rat. NAX-5055 is also active in the Frings audiogenic seizure model of reflex epilepsy. In contrast, NAX-5055 (up to 20 mg/kg) displays little to no anticonvulsant activity in the maximal electroshock or s.c. PTZ models of generalized epilepsy. NAX-5055 is well tolerated by animals with a TD_{50} of 21 mg/kg as estimated by the rotorod test. Thus, NAX-5055 displays a high therapeutic index.

Effect of NAX-5055 on the Rate of Corneal Kindling Acquisition

The overexpression of galanin within the CNS has been demonstrated to delay kindling acquisition rate in both mice and rats (Kokaia et al., 2001; Kanter-Schlifke et al., 2007). NAX-5055 displays potent anti-seizure activity against fully developed rat hippocampal kindled seizures and mouse corneal kindled seizures (Table 3.1). We hypothesized that NAX-5055, like galanin, would delay kindling acquisition. To test this hypothesis, we evaluated the ability of NAX-5055 to modify kindling acquisition in the corneal kindled mouse model of partial epilepsy (Matagne and Klitgaard, 1998). Of all the mice that received kindling stimulations, 87.5% (14/16) of vehicle-treated mice and 70.8% (17/24) of NAX-5055-treated mice reached a fully kindled state, i.e. 5 consecutive stage 5 seizures within 32 stimulations. Not all NAX-5055 treated mice became fully kindled (7/24); however, 91.7% (22/24) of mice treated with NAX-5055 displayed at least one generalized stage 5 seizure (Figure 3.1A). Analysis of the number of stimulations to reach each behavioral seizure stage failed to detect a difference in the

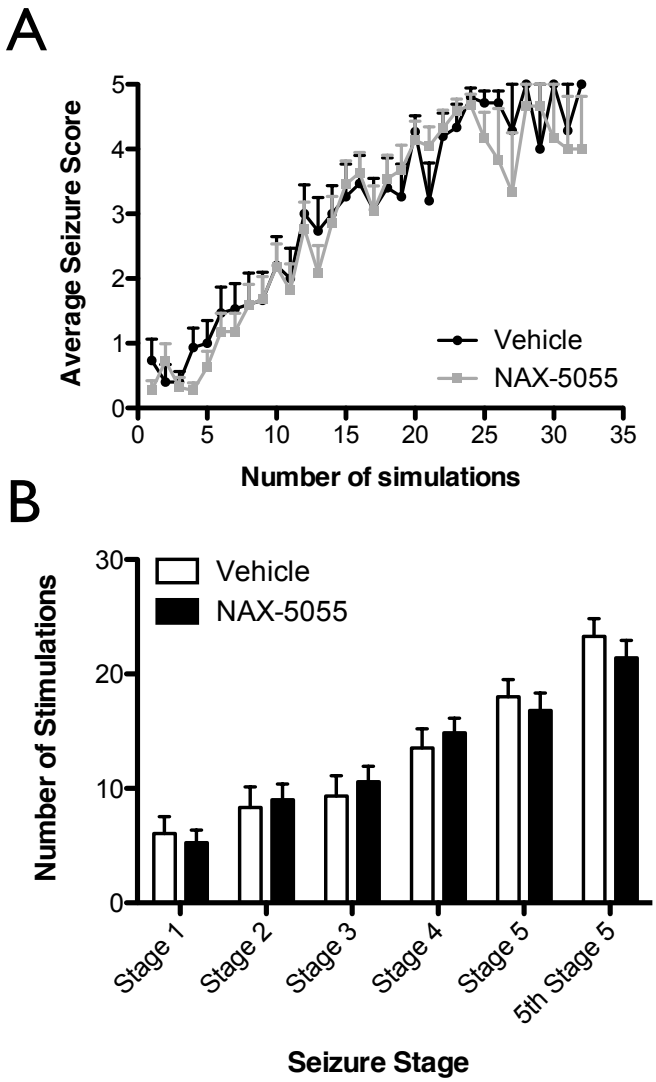


Figure 3.1. NAX-5055 did not affect the rate of corneal kindling acquisition in mice.

A.) Corneal kindling acquisition curves for vehicle- and NAX-5055-treated mice. All mice were stimulated twice daily with a subconvulsive corneal stimulation of 3mA for 3 sec. NAX-5055 (4 mg/kg) or vehicle was administered 1 hr before corneal stimulation. Seizures were scored according to the 5-stage Racine scale. Individual data points represent the average seizure score ± SEM following each stimulation.

B) Average number of stimulations to reach each seizure stage and the fully kindled state; i.e., five consecutive stage 5 seizures. No significant difference was seen between mice administered vehicle (n =16) or NAX-5055 (n = 24) (Two-way ANOVA, $p > 0.05$).

kindling acquisition rate between vehicle and NAX-5055 treated mice (Figure 3.1B, $p > 0.05$).

Anti-seizure Efficacy of NAX-5055 is Reduced with Repeated Systemic Administration

One hypothesis to explain the failure of NAX-5055 to attenuate kindling acquisition is that the efficacy of NAX-5055 is reduced following repeated systemic administration. To test this hypothesis, mice received an i.p. dose of NAX-5055 once daily for four consecutive days in the absence of any stimulation. One hour following the 4th and final injection of NAX-5055, mice were challenged in the 6 Hz psychomotor seizure test (Figure 3.2A). Mice that received a single systemic dose of NAX-5055 (4 mg/kg) displayed significant protection from the 6 Hz (32 mA) corneal stimulation (Figure 3.2B, n = 8 per group). In contrast, the anticonvulsant efficacy of NAX-5055 was markedly reduced in mice subjected to repeated systemic injections; e.g., only 25% of mice assigned to the repeated-dose group were protected in the 6 Hz test (Figure 3.2B, n = 8, per group).

The results shown in Figure 3.2B suggest that repeated systemic administration of NAX-5055 results in a robust decrease in antiseizure efficacy. There are a number of possible mechanisms that could contribute to this phenomenon. To begin to address this issue, we evaluated both pharmacodynamic and pharmacokinetic properties of NAX-5055 in the mouse. In this study we have focused on the role of the chemical modifications added to NAX-5055, the availability of galanin receptors, and the role of efflux transporters.

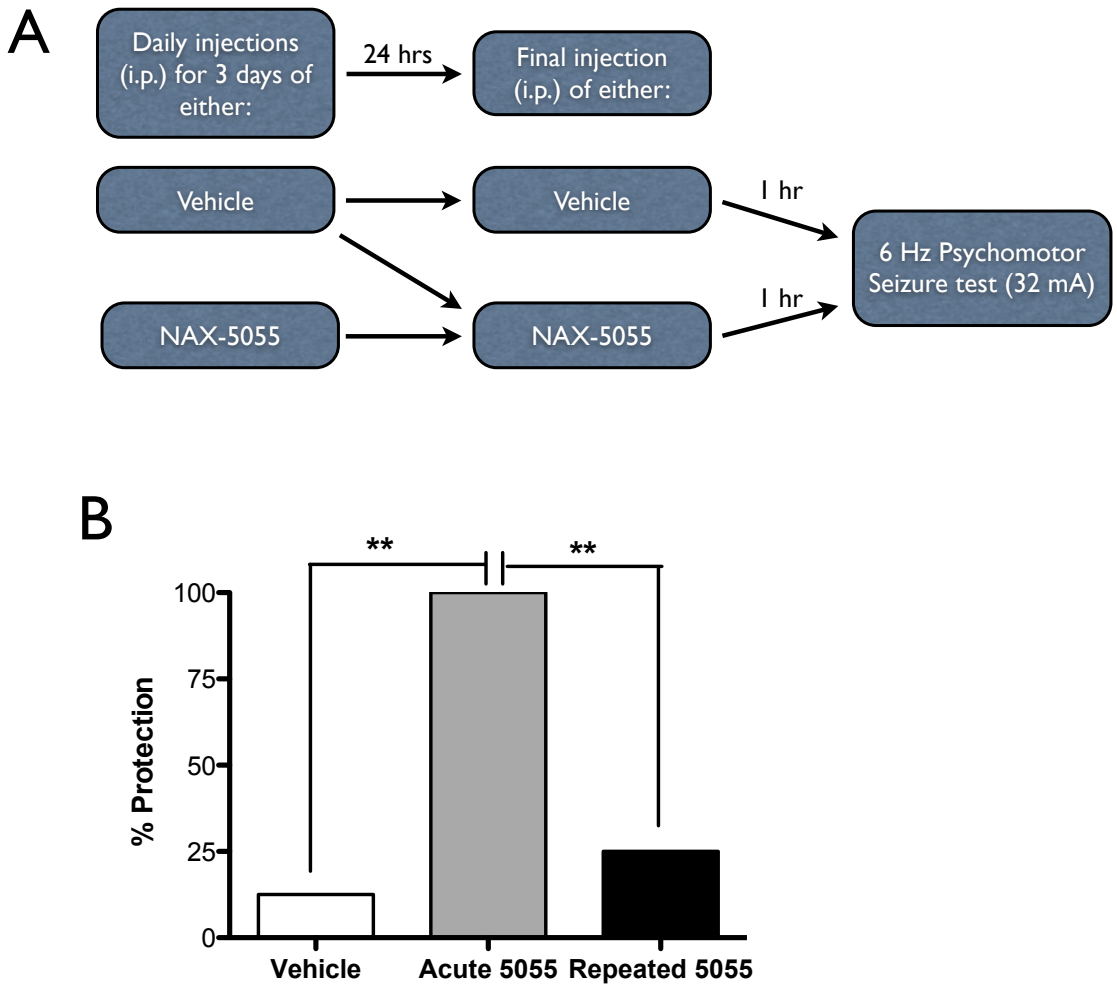


Figure 3.2. Repeated systemic administration of NAX-5055 reduces anti-seizure efficacy in the 6 Hz psychomotor seizure model.

A) Schematic of the experimental design. Mice were administered NAX-5055 (4 mg/kg, i.p.) or vehicle 1hr prior to a 6 Hz, 32mA corneal stimulation.

B) NAX-5055 displays significant anti-seizure activity following acute administration (i.e., acute 5055). In contrast, the anti-seizure efficacy of NAX-5055 is significantly reduced after four, once daily systemic injections (i.e., repeated 5055). Mice were scored as either protected (100%) or unprotected (0%) in response to the 6 Hz corneal stimulation. (**$p < 0.01$; Kruskal-Wallis test, $p = 0.001$; $n = 8$ per group).

NAX-5055 was designed around the active galanin fragment Gal (1-13), which displays high affinity for the galanin receptors (Lang et al., 2007). The chemical modifications made to this fragment were implemented to increase systemic availability (Bulaj et al., 2008; Robertson et al., 2011). Studies with a scrambled peptide 805-1 demonstrate that the galanin fragment is critical to the antiseizure activity of NAX-5055 in the 6 Hz test (White et al., 2009). The scrambled peptide 805-1 is identical to NAX-5055 with the exception that Try^2 and Trp^9 are swapped. These two residues are critical for the binding of galanin to its cognate receptors. Indeed, 805-1 displays weak binding to galanin receptors *in vitro* (data not shown). To determine if the active galanin fragment or chemical modifications were involved in the decreased efficacy of NAX-5055 we tested whether repeated dosing with 805-1 would modify the antiseizure activity of NAX-5055 (Figure 3.3A). As seen previously, a high percentage (75%) of mice were protected in the 6 Hz test with a single systemic 4 mg/kg dose of NAX-5055 (Figure 3.3B). In contrast, a low percentage (12.5%) of mice that received repeated doses of NAX-5055 (4 mg/kg, i.p.) were protected against the 6 Hz stimulation (Figure 3.3B). When mice were exposed to the scrambled peptide 805-1 once daily for three days and then treated with a single i.p. dose of NAX-5055 (4mg/kg) on the fourth and final day, 100% of mice were protected in the 6 Hz test (Figure 3.3B, n = 8 per group). The results of this study suggest that the integrity of the Gal(1-13) fragment of NAX-5055 is important to the reduced efficacy following repeated dosing.

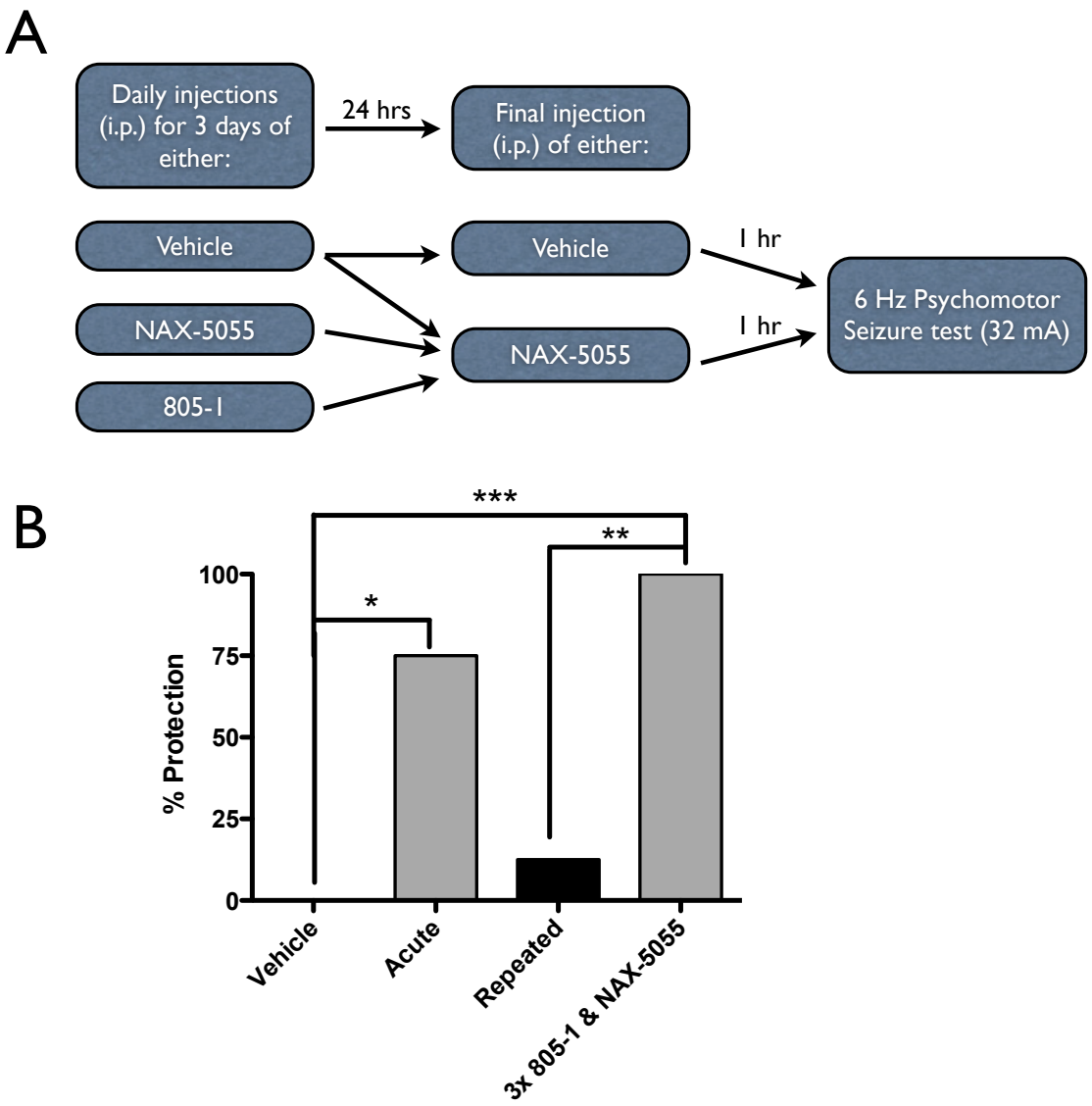


Figure 3.3. Repeated administration of the scrambled peptide 805-1 does not result in reduced efficacy of NAX-5055 in the 6 Hz psychomotor seizure model.

A) Schematic of the experimental design.

B) Bars represent the percentage of treated mice that were protected (i.e., showed no behavioral seizure activity) following a 6 Hz, 32 mA corneal stimulation. NAX-5055 and 805-1 were administered at 4 mg/kg, i.p. Acute administration of NAX-5055 displays significant anti-seizure activity (acute). In contrast, mice exposed to repeated daily administration of NAX-5055 displayed a markedly attenuated anti-seizure response to NAX-5055 (repeated). However, repeated daily administration of the scrambled peptide 805-1 did not negatively affect the efficacy of NAX-5055 (i.e., 3x 805-1 & NAX-5055). Kruskal-Wallis test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 8$ per group.

Central Administration of NAX-5055 following Repeated Systemic Administration Restores Anti-seizure Efficacy

Galanin receptors have been demonstrated to internalize following agonist exposure in immortalized cell lines (Xia et al., 2005; 2008). Once internalized the GalR1 receptor, the preferred target of NAX-5055, is trafficked to lysosomes and degraded (Xia et al., 2008). Thus, reduced efficacy to the systemic anti-seizure activity of NAX-5055 could result from a reduction in galanin receptor availability within the brain. To test this hypothesis, a group of 16 mice were administered NAX-5055 (4 mg/kg, i.p.) once daily for 3 days. On the fourth day, mice were randomly split into two groups. The first group received their fourth and final dose systemically (4 mg/kg, i.p.), whereas the second group received their final dose centrally (i.c.v.) (0.4 nmol/5μL). The anti-seizure activity of NAX-5055 was evaluated using the 6 Hz test (32 mA). Consistent with previous results (Figure 3.2B & 3.3B), mice that received repeated systemic injections of NAX-5055 demonstrated minimal protection (12.5% protection) in the 6 Hz test (Figure 3.4A). In contrast, a high percentage (i.e., 75% protection) of mice that received their final dose of NAX-5055 via the i.c.v. route were considered protected in the 6 Hz test (Figure 3.4B). Mice that received a single i.c.v. injection of NAX-5055 demonstrated complete seizure protection (Figure 3.4B). The demonstration that centrally administered NAX-5055 restores the anti-seizure effect in otherwise resistant mice suggests that galanin receptors remain available for interaction with NAX-5055, assuming the agonist can gain access to the receptors.

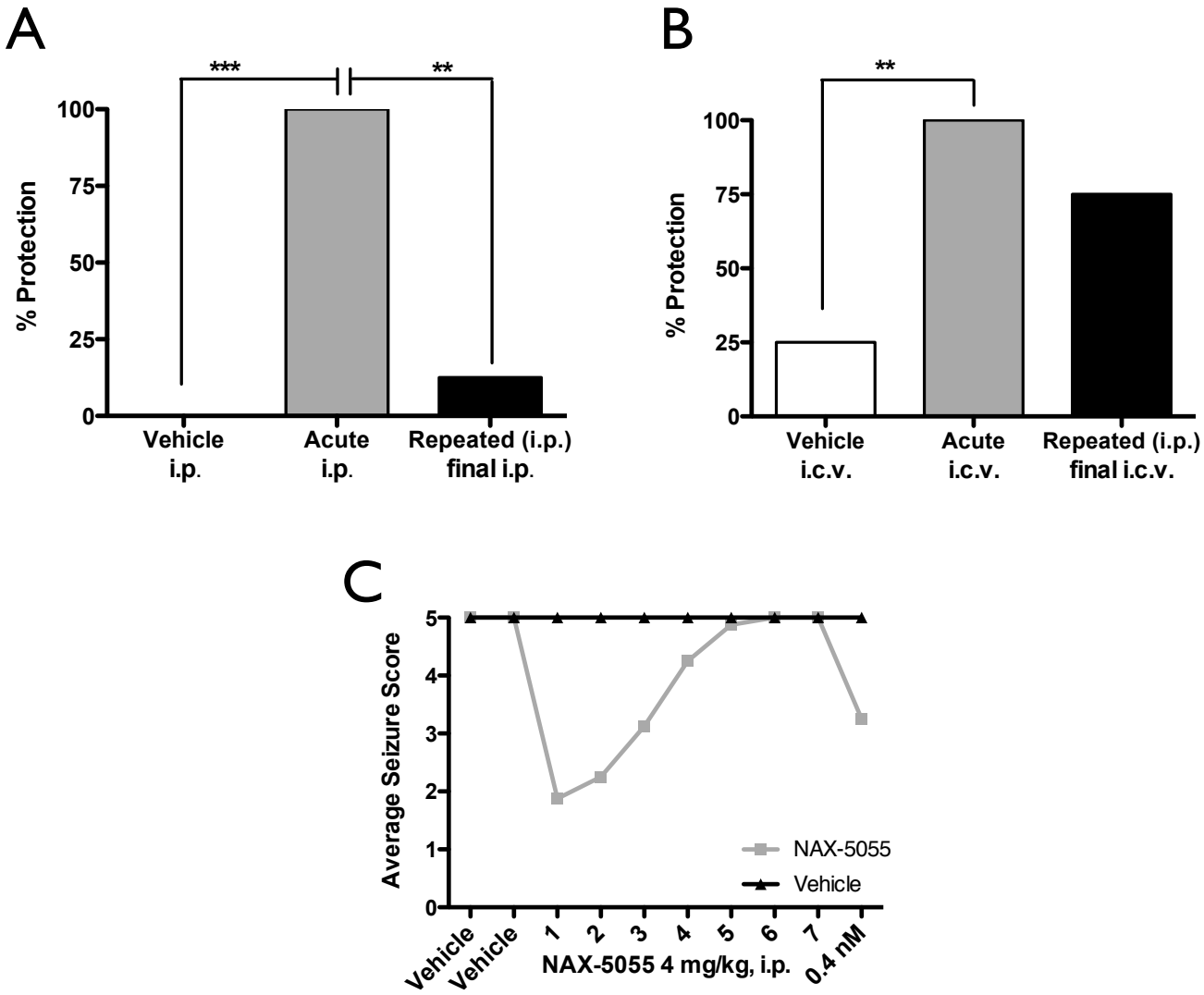
To confirm that these observations were not specific to the 6 Hz psychomotor seizure test, similar experiments were conducted in corneal kindled mice (Figure 3.4C).

Figure 3.4. Central administration of NAX-5055 elicits anti-seizure activity in mice subjected to repeated systemic NAX-5055 administration.

A) Mice were treated using the same dosing paradigm described in Figure 2A. NAX-5055 was administered at 4 mg/kg, i.p. Bars represent the percentage of mice protected from seizures in the 6 Hz (32 mA) test. Acute administration of NAX-5055 displays significant anti-seizure activity (grey bar). In contrast, repeated daily administration of NAX-5055 significantly attenuates anti-seizure activity (black bar). Kruskal-Wallis test, $^{**}p < 0.01$, $^{***}p < 0.001$, n = 8 per group.

B) Mice were treated using a similar dosing paradigm described in Figure 3.2A. However, the final injection of NAX-5055 (0.4 nmol/5µl) or vehicle was administered i.c.v. Acute administration of NAX-5055 displays significant anti-seizure activity (grey bar). When the final injection of NAX-5055 is administered i.c.v. in repeatedly treated mice, there is no attenuation of anti-seizure activity (black bar). Kruskal-Wallis test, $^{**}p < 0.01$, n = 8 per group

C) Corneal kindled mice were administered NAX-5055 (4 mg/kg) once daily for seven days. Initial NAX-5055 administration reduced the average seizure severity from a generalized stage 5 seizure to an average of a stage 2 seizure. The reduction in seizure severity was attenuated with every additional NAX-5055 administration and subsequent stimulation. However, partial anti-seizure activity was observed following i.c.v (0.4 nmol/5µL) injection in mice resistant to the systemic effects of NAX-5055. Mice given only vehicle injections displayed stable stage 5 seizure activity for the duration of the experiment (n = 8 per group).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0.4 nM
NAX-5055 4 mg/kg, i.p.

Mice were fully kindled and confirmed to display consistent stage 5 seizure activity prior to initiating treatment with NAX-5055 (4 mg/kg) or vehicle i.p.. Control mice that received vehicle injections throughout the study displayed stable stage 5 seizures in response to daily stimulations. Following the first injection of NAX-5055, 62.5% (5/8) of corneal kindled mice were protected and displayed no behavioral seizure activity (stage 0), while the remaining 37.5% (3/5) mice were not protected and displayed stage 5 seizures (Figure 3.4C). Following each additional injection of NAX-5055 and stimulation, the number of animals displaying protection from seizure activity decreased. By the 5th day of systemic NAX-5055 administration all mice had become resistant to the anti-seizure effects of NAX-5055 and displayed stage 5 seizures following stimulation. However, the subsequent i.c.v. injection of NAX-5055 (0.4 nmol/5μL) to this group partially recovered the anti-seizure activity of NAX-5055. Fifty percent of the previously nonprotected mice displayed reduced seizure severity (stage 1-3 seizures), while the other 50% remained resistant and displayed stage 4/5 seizures (Figure 3.4C, n = 8 per group). These results suggest that the reduced anti-seizure efficacy of NAX-5055 is not model specific and supports the conclusion that central galanin receptors are available to be engaged if the agonist can gain access to the receptors.

In order to confirm that previous results are not specific to a single dose of NAX-5055, dose-response studies were conducted. To generate dose-response curves, mice were treated with a single acute dose or repeated (i.e., once daily for three consecutive days) doses of NAX-5055. Similar to previous experiments, the final injection was administered either systemically (i.p.) or centrally (i.c.v.) and mice were then subjected to the 6 Hz test. The dose of NAX-5055 was varied to obtain a dose response. As shown in

Figure 3.5A, a marked shift to the right in the dose-response curve was noted in those mice that received repeated systemic doses of NAX-5055 for 3 days followed by a final systemic challenge dose of NAX-5055. Despite the increase in dose needed to reach 100% protection in the repeatedly treated mice (up to 20 mg/kg) no significant motor toxicity was observed (data not shown). In contrast, no significant shift in the dose-response curves was observed when the final NAX-5055 dose was administered i.c.v. (Figure 3.5B). In this study, some mice displayed a loss of righting reflex at the highest i.c.v. dose tested (i.e. 4 nmol/5μL) and were excluded from analysis because their response to stimulation in the 6 Hz test could not be discerned. The ED_{50} and 95% confidence intervals for both of these studies are summarized in Table 3.2.

Transport of NAX-5055

Of the myriad of possible mechanisms that could account for the notable reduction in efficacy to NAX-5055 following repeated systemic administration, efflux transport out of the brain is a possible mechanism. The ABC efflux transporter P-gp is well documented to remove xenobiotic compounds from the brain (Aronica et al., 2011). To evaluate whether P-gp could transport NAX-5055, we tested the substrate activity of NAX-5055 for the two isoforms of P-gp in mice, mdr1a and mdr1b. To transport compounds across membranes, P-gp requires energy in the form of adenosine triposphate (ATP) (Sauna and Ambudkar, 2007). Interaction of P-gp with a substrate compound results in the cleavage of ATP to ADP and the release of a free inorganic phosphate group, which can be measured using a colorimetric assay (Sarkadi et al., 1992). Incubation of NAX-5055 with either mdr1a or mdr1b did not increase the accumulation

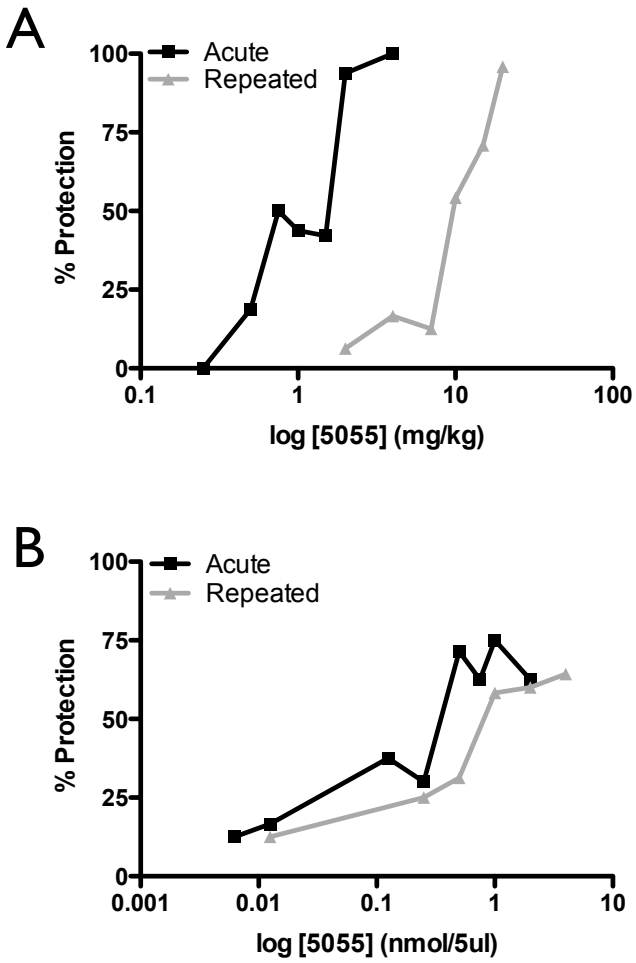

Figure 3.5. Dose response curves following acute and repeated NAX-5055 treatment.

A) Dose response curves for mice administered acute or repeated systemic injections of NAX-5055. Results are plotted as the percentage of mice protected from seizure activity in the 6 Hz (32mA) test. The repeated-treatment, dose-response curve displays a significant rightward shift. This suggests a decrease in potency of NAX-5055 following repeated administration (n = 8-16 per dose per group).

B) Dose response curves for mice receiving NAX-5055 i.c.v. Repeatedly dosed mice received three, once daily systemic NAX-5055 injections (4 mg/kg, i.p.) prior to a final i.c.v. injection. Results are plotted as the percentage of mice protected from 6 Hz (32 mA) seizures. No significant shift between the acute and repeated dose response curves was observed (n = 8-16 per dose per group).

Table 3.2

ED_{50} and 95% confidence intervals following systemic or central administration of NAX-5055 in acute or repeatedly dosed mice

Treatment Group	ED_{50} (mg/kg)	95% Confidence Interval
I.P. administration		
Acute	1.1	0.85 – 1.38
Repeated	8.8	7.05 – 10.75
I.C.V. administration		
Acute	0.3	0.13 – 0.63
Repeated	1.25	0.36 – 4.98

Treatment groups were defined by the route of administration (i.e., i.p. or i.c.v.) by which mice received NAX-5055 prior to testing in the 6 Hz (32 mA) model. Within each group, mice are separated into those that received only one dose of NAX-5055 on the day of testing (acute) and those that received three once daily injections of NAX-5055 (repeated) prior to the test day. (See methods for details)

of free phosphate groups above background levels (Figure 3.6A). In contrast, incubation of mdr1a and mdr1b with verapamil (60 μM), a known substrate for Pgp, resulted in increased phosphate accumulation above baseline levels; thereby confirming assay validity (Figure 3.6A). Thus it seems unlikely that efflux transport of NAX-5055 mediated by P-gp could account for the reduced efficacy of NAX-5055.

Discussion

Our initial objective for this study was to evaluate the effect of NAX-5055 on the rate of corneal kindling acquisition. When NAX-5055 failed to affect kindling acquisition rate, we investigated the extent to which repeated systemic administration of NAX-5055 would affect its antiseizure efficacy. It quickly became evident that the anti-seizure potential of NAX-5055 was markedly reduced following repeated systemic administration. As a result, the focus of this study shifted to understanding the potential pharmacokinetic and/or pharmacodynamic mechanism(s) that could account for this phenomenon.

When administered 1 hr prior to each corneal stimulation NAX-5055 (4 mg/kg, i.p.), failed to affect the rate of corneal kindling acquisition in mice (Figure 3.1). The failure of NAX-5055 to exhibit any effect on kindling acquisition was surprising. In previous studies, when galanin is overexpressed in the CNS a significant delay in the appearance of generalized stage 4/5 seizures in both mouse and rat hippocampal kindling models was observed (Kokaia et al., 2001; Kanter-Schlifke et al., 2007). Further, galanin has been suggested to facilitate the inhibitory action of low-frequency stimulation on

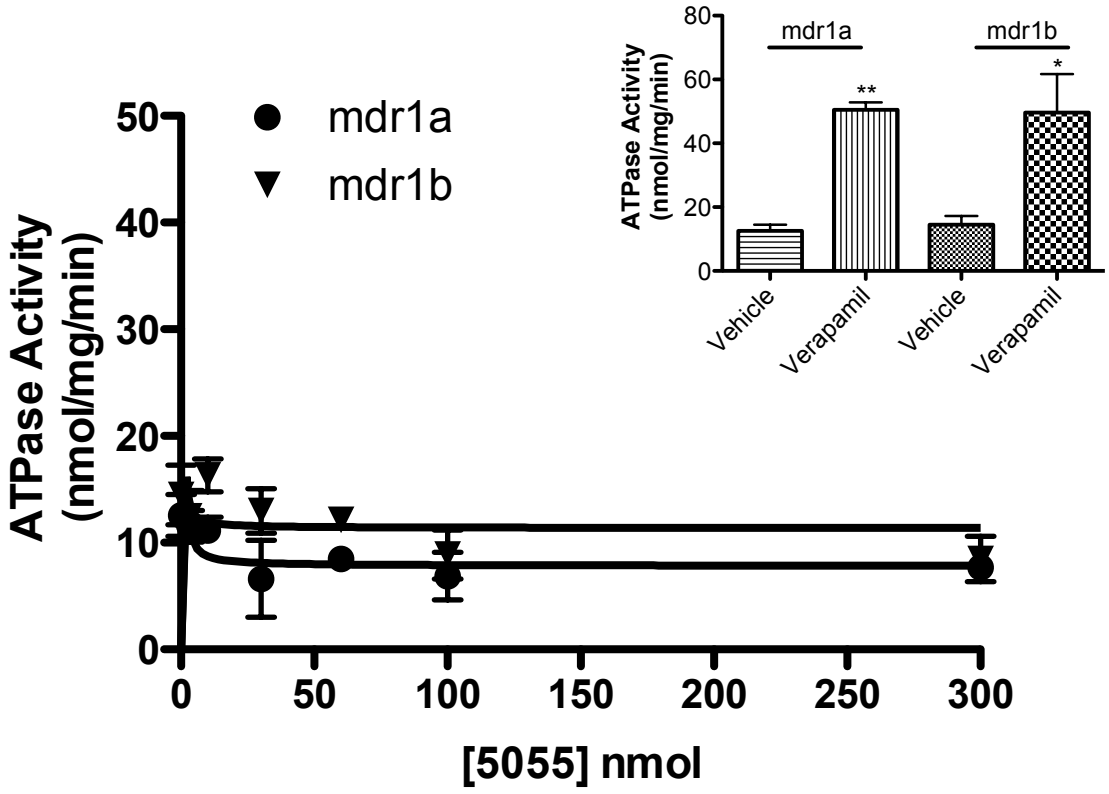

Figure 3.6. NAX-5055 is not a substrate for the blood brain barrier transporter p-glycoprotein.

A.) No ATPase activity was observed following incubation of NAX-5055 with either mouse P-gp isomer mdr1a or mdr1b. Results are plotted for the activity of ATPase vs. concentration of NAX-5055. Calculated ATPase activity for all concentrations of NAX-5055 was lower than those observed in the presence of buffer alone. Assays were run in quadruplicate for each isoform. (Inset). The p-glycoprotein substrate verapamil (60 μM) was assayed in tandem with NAX-5055 as a positive control and displayed a consistent increase in ATPase activity in all assays (Paired t-test, *$p < 0.05$, **$p < 0.01$)

kindling acquisition. Low-frequency stimulation (1 Hz) of the perforant path inhibits the development of stage 4/5 seizures in rats (Mohammad-Zadeh et al., 2007). When the non-specific galanin antagonist M35 was injected into the hilus during low-frequency stimulation this effect was attenuated, suggesting galanin is important for the anti-kindling effects observed with low frequency stimulation (Sadegh et al., 2007). In addition, NAX-5055 displays potent antiseizure activity against fully expressed mouse corneal kindled and rat hippocampal kindled seizures (White et al., 2009). This activity suggests that NAX-5055 is capable of modulating neuronal circuits considered important for generating kindled seizures. Considering that galanin displays anti-kindling activity and NAX-5055 can block fully kindled seizures; one possible explanation for the failure of NAX-5055 to modulate corneal kindling is that repeated administration reduces the efficacy of NAX-5055.

To investigate the effect of repeated NAX-5055 administration, mice were treated daily for three days with NAX-5055 (4 mg/kg, i.p.) in the absence of any stimulation. Following the 4th and final dose of NAX-5055 mice were tested for seizure protection in the 6 Hz psychomotor seizure model. This dosing paradigm resulted in a marked reduction in antiseizure efficacy of NAX-5055 (Figure 3.2). A similar response was observed in C57BL/6 mice in the 6 Hz model, suggesting this phenomenon is not strain specific (data not shown). Further, in fully kindled mice, a reduction in seizure severity was observed following a single 4 mg/kg dose of NAX-5055. However, this effect was rapidly attenuated with each additional NAX-5055 administration (Figure 3.3C), suggesting the observed reduction in NAX-5055 efficacy is not model specific. Together

these studies support the hypothesis that the anti-seizure efficacy of NAX-5055 is reduced as a result of repeated systemic administration of an acutely efficacious dose.

To determine the contribution of the active Gal(1-13) fragment and the chemical modifications toward the decrease in NAX-5055 efficacy, we utilized a scrambled structural isomer 805-1 where amino acid residues Trp^2 and Tyr^9 were swapped maintaining the chemical modifications of NAX-5055. These two amino acid residues are known to be critical for the binding of galanin with its receptors (Land et al., 1991; Kask et al., 1998; Lundström et al., 2005). Binding studies with 805-1 confirm that this peptide lacks an interaction with galanin receptors (data not shown). Further, when administered acutely, 805-1 is not anticonvulsant in the 6 Hz model (White et al., 2009). Once-daily systemic injections of 805-1 for 3 consecutive days did not negatively affect the efficacy of acutely administered NAX-5055 (4 mg/kg) in the 6 Hz test (Figure 3.3). This observation suggests that the modifications added to the active galanin fragment of NAX-5055 are most likely not responsible for the reduction in NAX-5055 efficacy observed in this study.

Although our results do not fulfill the clinical definition of drug-resistant epilepsy (Kwan et al., 2010a); we have used the hypotheses proposed to explain this phenomena to guide the design of studies aimed at elucidating the mechanism(s) underlying the observed reduction in efficacy following repeated NAX-5055 administration. The two main hypotheses for drug-resistant epilepsy involve the pharmacodynamic mechanisms of decreased target availability and increased efflux transport (Kwan and Schachter, 2011). We have also considered a pharmacokinetic mechanism of increased peripheral metabolism of NAX-5055, discussed in Chapter 4.

The target hypothesis of drug-resistant epilepsy postulates that there is an alteration in the sensitivity of cellular target(s) toward the activity of antiepileptic drugs as a result of chronic treatment (Remy and Beck, 2006). Evidence supporting this hypothesis includes the alteration of sodium channel function in resected tissue from drug-resistant patients, polymorphisms in sodium channels, and alteration in $GABA_A$ receptor subunit composition (Remy et al., 2003; Kwan et al., 2008; Loup et al., 2009). G-protein coupled receptors are known to be subject to complex intracellular trafficking and down-regulation following repeated agonist exposure in neurons (Bernard et al., 2006). Following galanin exposure, both GalR1 and GalR2 receptors are internalized in immortalized cell lines (Wang et al., 1998b; Xia et al., 2005; 2008). GalR1 receptors, the assumed primary target of NAX-5055, are further trafficked to lysosomes for degradation, thereby reducing their membrane expression (Xia et al., 2008). Thus, central galanin receptor modulation is a likely hypothesis for the reduced efficacy of NAX-5055 following repeated administration.

We hypothesized that if central galanin receptors were altered due to a lack of availability or decreased sensitivity to NAX-5055, then the attenuated antiseizure activity would persist following central administration. However, if central galanin receptors were unaltered by repeated systemic injection, then NAX-5055, when administered directly into the brain, should inhibit seizures in mice resistant to the systemic effects. We tested this hypothesis in both the 6 Hz psychomotor seizure model and the corneal kindled mouse. In both partial seizure models, central administration of NAX-5055 was successful in blocking seizures in mice rendered resistant to the systemic effects of 4

mg/kg NAX-5055 (Figure 3.4). This observation suggests that central galanin receptors are unaltered in mice resistnat to systemically administered NAX-5055.

Dose-response experiments were conducted to further test the observation that galanin receptors were unaltered by repeated dosing. When all injections of NAX-5055 were administered systemically, a significant rightward shift in the dose-response curve was observed. In contrast, no significant shift in the dose response was observed when the final injection of NAX-5055 was administered directly into the lateral ventricles (Figure 3.5). Together these data suggest that repeated systemic administration of NAX-5055 results in a reduction in potency, but not efficacy; i.e., complete seizure control (maximum efficacy) could be obtained by increasing the administered dose. In addition, the lack of a shift in the central administration, dose-response curve supports the conclusion that galanin receptors remain available following repeated systemic injections. Thus it seems unlikely that alteration in galanin receptor function can account for the reduced potency associated with repeated NAX-5055 administration.

The second hypothesis of drug-resistant epilepsy is the ‘transporter hypothesis’. This hypothesis proposes that drug resistance results from overexpression of multidrug efflux transporters within, or around, the epileptic focus (Remy and Beck, 2006; Kwan and Schachter, 2011). We attempted to determine whether multidrug efflux transporters contributed to the reduced anti-seizure activity of NAX-5055 following repeated systemic administration. This hypothesis is of particular interest in light of the decreased potency of NAX-5055 observed in dose-response studies. An increase in efflux transport of NAX-5055 could underlie the shift in potency seen with repeated NAX-5055 (Miller et al., 2008).

ATPase-binding cassette (ABC) transporters are a large family of transmembrane proteins that transport a wide range of substrates, including some antiseizure drugs, across cellular membranes (Luna-Tortós et al., 2008; Aronica et al., 2011; Kwan and Schachter, 2011). The most extensively studied ABC transporter is P-glycoprotein, a key element in the blood brain barrier. P-gp is expressed in capillary endothelial cells where it acts to transport xenobiotics out of the brain, thereby reducing the concentration of substrate drugs (Aronica et al., 2011; Kwan and Schachter, 2011). In patients with drug-resistant epilepsy, there is a large body of evidence demonstrating that P-pg expression is increased in brain capillaries. In addition, aberrant expression of P-gp is observed on astrocytes that constitute the blood-brain barrier (Aronica et al., 2003; Kwan et al., 2010b). In the mouse, P-gp is encoded by two genes, mdr1a and mdr1b, and both are highly expressed at the blood brain barrier (Schinkel et al., 1994; Rizzi et al., 2002). It is of particular interest that P-gp has been shown to actively transport lipophilic drugs that would be predicted to cross membranes by passive diffusion (Löscher and Potschka, 2005). Thus it seems possible that NAX-5055 could enter the brain by passive diffusion, but still be subject to increased efflux transport via interaction with P-gp.

To evaluate any interaction between NAX-5055 and P-gp we utilized an *in vitro* substrate assay. In this assay verapamil, a known P-gp substrate, increases ATPase activity in both isoforms of mouse P-gp (mdr1a & mdr1b). In contrast, NAX-5055, at concentrations up to 300nM, failed to affect baseline ATPase activity (Figure 3.6). Thus, NAX-5055 is unlikely to act as a substrate for Pgp.

Despite the inability of Pgp to transport NAX-5055, we cannot completely rule out the contribution of transporters to the reduced potency of NAX-5055. Other ABC

transporters including the multidrug resistance-associate proteins and breast cancer resistance protein, as well as non-ABC transporters have also been implicated in drug resistance (Aronica et al., 2011) and may actively transport NAX-5055. Additional work is necessary to determine if one or more of these transport systems is capable of regulating brain levels of NAX-5055.

A final hypothesis to explain the reduced potency of NAX-5055 observed following repeated systemic administration is an increase in peripheral metabolism of NAX-5055. The most direct way to address this hypothesis is to measure brain and plasma concentrations of NAX-5055 following acute and repeated systemic administration. However, despite considerable effort, we have been unable to reliably quantify whole brain concentrations of NAX-5055 utilizing analytical chemistry. However, preliminary work utilizing LC/MS/MS techniques demonstrated that NAX-5055 is present in plasma samples of both acute and repeatedly treated mice one hour after systemic administration (data not shown). These data suggest that NAX-5055 remains intact following repeated systemic administration, but further work is required to quantitatively determine NAX-5055 concentrations.

To directly address the possibility of increased metabolism of NAX-5055 we assayed the response of peripheral galanin receptors following repeated systemic administration. Galanin receptors are expressed on β-cells of the pancreas, and activation of these receptors has been shown to inhibit insulin release (Gregersen et al., 1991; McDonald et al., 1994; Barreto et al., 2011). If NAX-5055 were subject to increased metabolism, we would expect to see an attenuation of the insulin response following repeated systemic administration. There was no significant difference in insulin levels

following acute or repeated systemic NAX-5055 administration (see Chapter 4). Thus, it seems unlikely that peripheral metabolism of NAX-5055 is directly responsible for the reduction in efficacy of NAX-5055 following repeated administration.

NAX-5055 could be inactivated once it crosses the blood brain barrier. Galanin has been shown to be inactivated by endogenous peptidases or metalloproteasees within the brain (Bedecs et al., 1995). These enzymes may directly target the active galanin fragment of NAX-5055, thereby inhibiting its interaction with galanin receptors. Although this mechanism warrants further study, it seems unlikely given the observation that the efficacy of NAX-5055 is unaltered following i.c.v. administration in repeatedly dosed mice. In addition, anti-seizure activity has been observed in mice overexpressing galanin, suggesting that chronically high galanin levels within the CNS do not attenuate peptide activity (McCown, 2006).

The initial goal of this study was to determine the disease modifying potential of NAX-5055. However, the focus of this study shifted to understanding the mechanism(s) of reduced NAX-5055 potency. It is tempting to speculate that the failure of NAX-5055 to affect kindling rate results from the shift in potency of NAX-5055 following repeated administration. Future studies utilizing chronic i.c.v. administration of NAX-5055 in addition to *in vitro* assays will continue to evaluate the disease-modifying potential of galanin receptor activation.

In conclusion, we have shown that NAX-5055 is a systemically available galanin analog with potent acute antiseizure activity that is subject to rapid attenuation following repeated systemic administration. Throughout the course of this study, we have shown that the reduced potency of NAX-5055 is not likely due to an alteration in galanin

receptor function, efflux transport by P-glycoprotein, or increased peripheral metabolism. As a result, it remains unclear what underlying mechanism(s) might account for the altered response to NAX-5055 observed in these studies. However, this phenomenon offers an interesting model to increase our understanding of the regulation of galanin receptors and galanin-based analogs *in vivo*. In addition, these studies will help direct the development of future galanin-based therapeutics.

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CHAPTER 4

THE SYNTHETIC GALANIN ANALOG, NAX-5055 INHIBITS INSULIN RELEASE AND INDUCES HYPERGLYCEMIA AFTER ACUTE AND REPEATED TREATMENT

Abstract

The neuropeptide galanin is widely expressed in both the central and peripheral nervous systems. However there is limited understanding of how each galanin receptor (GalR1, 2, and 3) mediates the physiological activity of galanin *in vivo*. To address this issue we have developed a systemically available, metabolically stable galanin analog, NAX-5055. NAX-5055 displays a preference for GalR1 receptors and possesses a potent anticonvulsant activity in animal seizure and epilepsy models, suggesting that NAX-5055 engages central galanin receptors. To determine if NAX-5055 also modulates the activity of peripheral galanin receptors, we evaluated the effect of NAX-5055 on glucose and insulin levels, which can be regulated by galanin receptors located on pancreatic β-cells. Both acute and repeated systemic administration of NAX-5055 (4 mg/kg) significantly increased blood glucose levels in NAX-5055-treated mice compared to those observed in vehicle-treated mice. No hyperglycemia was observed when amino acid residues in the active galanin fragment of NAX-5055, thought to critical for receptor binding, i.e., Trp^2

and Tyr^9, were reversed. NAX-5055 also alters the physiological response to a glucose challenge in mice. During a glucose tolerance test (GTT), mice administered either acute or repeated injections of NAX-5055 displayed impaired glucose handling and reduced insulin response to an acute glucose challenge. Here we have shown that systemic administration of a centrally active GalR1-preferring galanin analog produces acute hyperglycemia and an inhibition of insulin release *in vivo* and that these effects are not attenuated following repeated administration. NAX-5055 thus provides a powerful new pharmacological tool to further the understanding of function of peripheral galanin receptors *in vivo*.

Introduction

Galanin is a 29 (30 in humans) amino acid neuropeptide widely expressed throughout the central and peripheral nervous system with similar immunoreactivity in mice, rats, and humans (Tatemoto et al., 1983; Köhler and Chan-Palay, 1990; Lang et al., 2007). Immunohistochemical studies have shown that galanin colocalizes with a number of classical neurotransmitters including glutamate, acetylcholine, serotonin, and GABA. Due to this broad expression pattern there has been diverse interest in the physiology of galanin. To date galanin has been linked to the regulation of a number of neuronal functions including learning and memory, neuronal excitability, neuroprotection, and neuroendocrine regulation (Mazarati et al., 1998; Crawley, 2008; Hobson et al., 2008). The ability of galanin to modulate these physiological processes has led to an interest in the development of galanin-based therapies for a number of common pathological states including epilepsy, neuropathic pain, Alzheimer's disease, and diabetes (Kask et al.,

1997; Crawley, 2008; Lerner et al., 2008; Mechenthaler, 2008). Galanin is known to bind to three distinct G-protein coupled receptors GalR1, GalR2, and GalR3 (Branchek et al., 2000). Activation of either the GalR1 or GalR3 receptor results in a generally inhibitory action on the cell due to selective coupling to the inhibitory G-protein G_i (Parker et al., 1995). In contrast, GalR2 receptors demonstrate a more complicated signaling cascade due to their interaction with a number of different G-proteins, including G_i and $G_{q/11}$, resulting in either a inhibitory or excitatory cellular response (Lang and Kofler, 2011). The understanding of how each of these receptor subtypes contributes to the different physiological actions of galanin has been limited by the lack of systemically active, receptor-selective, galanin agonists and reliable galanin receptor antibodies (Lu and Bartfai, 2009; Robertson et al., 2011). The development of better tools to investigate the effects of galanin both *in vivo* and *in vitro* will facilitate the potential development of neuropeptide-based therapeutics.

Previous attempts to generate systemically active galanin agonists have produced two compounds: galmic and galnon (Saar et al., 2002; Bartfai et al., 2004). However, both compounds were shown to interact with nongalanin receptors in the micromolar (μM) range thereby limiting their use as selective pharmacological tools (Lu et al., 2005). To address this issue, Bulaj and colleagues successfully designed a series of truncated galanin analogs in which nonessential amino acid residues are replaced by cationic and/or lipoamino acid residues (Bulaj et al., 2008). One analog from this group, NAX-5055, was demonstrated to possess a 15-fold preference for GalR1 over GalR2 with nanomolar (nM) binding affinity in a time-resolved fluorescence binding assay (Bulaj et al., 2008; White et al., 2009).

Although the galaninergic system provides an interesting set of molecular targets for therapeutic development, modulation of this system is complicated by the broad physiological activity of the neuropeptide. As a result, it is important to increase our understanding of how the different galanin receptor subtypes mediate the activity of galanin in a region and cell-type specific manor. Of particular interest is the observation that GalR1 mRNA and immunoreactivity are highly expressed in both the ventral hippocampus and β cells of the islets of Langerhans (Parker et al., 1995; Gustafson et al., 1996; Jungnickel and Gundlach, 2005; Bhandari et al., 2010). Within the ventral hippocampus galanin has been show to potently inhibit the release of neurotransmitters including acetylcholine and glutamate; thus suggesting a role for galanin in mediating cellular excitability in a region known to generate seizure activity (Zini et al., 1993; Mazarati et al., 2000; Yoshitake et al., 2011). Increasing the concentration of central galanin through either direct injection or genetic overexpression delays acquisition of kindling and reduces the severity and duration of seizures following electronically or chemically evoked status epilepticus (Schlifke et al., 2006; Kanter-Schlifke et al., 2007). Further, we have recently shown that the GalR1 preferring analog NAX-5055 has potent anticonvulsant activity in a number of seizure and epilepsy animal models, suggesting that the GalR1 receptor is a promising molecular target for seizure control (White et al., 2009).

In the peripheral nervous system, galanin is known to regulate insulin secretion and blood glucose levels in may species including rodents and canines (McDonald et al., 1994; Ahrén, 2000). Galanin is expressed in sympathetic nerve terminals that innervate the endocrine pancreas and is colocalized with norepinephrine (Mei et al., 2006).

Stimulation of the mixed pancreatic nerve bundle results in the release of galanin at sufficient levels to inhibit insulin secretion in canines (Dunning and Taborsky, 1989). Further, galanin has been shown to reduce glucose-stimulated insulin release from cultured β islet cells of the pancreas (Gregersen et al., 1991). Consistent with the inhibition of insulin secretion, galanin infusion increases blood glucose levels in canines, but not in humans (Dunning and Taborsky, 1989; McDonald et al., 1994). Although this hyperglycemic response could be mediated by the reduction of insulin secretion, there is additional evidence that glucagon levels are also increased following galanin infusion, which may play a role in the effect of galanin on circulating glucose levels (Dunning et al., 1986; Dunning and Taborsky, 1989). Recent work investigating the expression levels of the galanin receptors on mouse β-cells showed detectable mRNA levels for all three receptors, with GalR1 demonstrating the highest expression levels (Barreto et al., 2011). However, it still remains unclear how the different galanin receptors mediate the galanin response in the endocrine pancreas. Previous attempts to use galanin-based pharmacology to study the effects of galanin on the endocrine pancreas have failed due to lack of specificity and off-target effects of the available pharmacological tools galnon and galmic (Quynh et al., 2005).

In this study we sought to determine if targeting GalR1 receptors with the GalR1-preferring agonist NAX-5055 would affect glucose and insulin regulation at doses that reliably produce anticonvulsant activity.

Methods

Animals

Adult male CF-1 mice weighing at least 18g (Charles River, Kingston, WA) were used for all experiments in this study. Animals were housed in a temperature-, humidity-, and light-controlled (12 hr light:dark cycle) facility. For all studies not involving a food restriction, mice were group-housed and provided free access to food (LabDiet) and water. For studies involving a fast, mice were initially group-housed and given full access to food and water until the beginning of the fasting period. At the beginning of the fasting period mice were housed individually and food restricted for 6 hr. During the fast, mice were allowed free access to water. All experimental procedures were performed in accordance with the guidelines established by the National Institutes of Health and received approval from the University of Utah's Animal Care and Use Committee. At the completion of all experimental procedures mice were humanly sacrificed by CO_2 asphyxiation.

Experimental Design and Dosing Regimen

Peptide Design and Preparation

The galanin analog NAX-5055 was originally designed and synthesized at the University of Utah as previously described (Bulaj et al., 2008), and later synthesized in bulk by NeoMPS (San Diego, CA). An inactive scrambled analog of NAX-5055, i.e. 805-1 in which Tyr^2 and Trp^9 were reversed, was also designed and synthesized at the University of Utah. Solutions were prepared by dissolving synthesized peptides in 0.9% saline containing 1% Tween 20 (Sigma-Aldrich, St. Louis, MO). Solutions were made

fresh daily. Prior to each experiment, peptide concentration was confirmed by UV absorbance of tyrosine and tryptophan residues (Cary 50 Bio UV Spectrophotometer, Varian). Dosing solutions containing peptide were administered to mice via intraperitoneal (i.p.) injection in a volume of 0.01 mL/g body weight.

For all studies mice were randomly selected from their home cage and placed into experimental groups with no intentional bias. Mice were removed from the colony, weighed, treated, and returned to the colony (in their home cage) or sacrificed between the hours of 8:00 AM and 5:00 PM. For the multiple dose experiments mice were administered a single i.p. injection of either NAX-5055 (4 mg/kg), 805-1 (4 mg/kg) or vehicle (0.9% saline with 1% Tween 20) once daily for 4 consecutive days. Whether peptide or vehicle was injected each day was dependent on the randomly assigned experimental group as described below. Glucose and/or insulin levels were obtained one hour post-peptide injection, corresponding to the time to peak effect (TPE) for anticonvulsant activity (Bulaj et al., 2008).

Repeated Administration of NAX-5055 and 805-1.

In experiments comparing repeated treatment with either NAX-5055 or 805-1, mice were split into one of four treatment groups (n = 8, per group). Group 1 (vehicle) mice received vehicle for all 4 days. Group 2 (acute NAX-5055) mice received vehicle for the first 3 days followed by a single injection of NAX-5055 on the 4^{th} day. Group 3 (repeated NAX-5055) mice received NAX-5055 for all 4 days. Group 4 (805-1) mice were treated with 805-1 for the first 3 days followed by a single injection of NAX-5055

on the 4th day. One hour after the last injection on day 4 glucose levels were obtained for all animals in all four groups.

Acute and Repeated Administration of NAX-5055

In experiments comparing the effect of acute or repeated treatment with NAX-5055 mice were split into one of three groups. Group 1 (vehicle) mice were administered vehicle for all 4 days. Group 2 (acute NAX-5055) mice were administered vehicle for the first 3 days followed by a single injection of NAX-5055 on the 4th day. Group 3 (repeated NAX-5055) mice were administered NAX-5055 for all 4 days. Mice treated with this dosing regimen were also subjected to a glucose tolerance test (described in detail below) wherein glucose and/or insulin levels were determined. Given the amount of blood (approximately 200 μL per animal) that was required to obtain enough plasma to run duplicate samples using an insulin ELISA, blood samples were taken from different sets of mice and then pooled for insulin determination. For determination of glucose levels, six animals were used in the vehicle and acute NAX-5055 treatment groups, eight animals were used from the repeated NAX-5055 treatment group and all animals were subjected to a GTT on the same day. For determination of insulin levels, 16 mice per group were used. Two separate glucose tolerance tests were conducted using eight animals per group per day. There was no significant difference in insulin levels from vehicle-treated mice so data from both days were pooled.

Glucose and Insulin Measurements

Blood samples were obtained from a cut made approximately 1 cm from the tip of the tail in gently restrained mice. Glucose levels were measured using a Bayer Contour blood glucose meter (9545C, Tarrytown, NY) and Contour blood glucose test strips (7098C, Tarrytown, NY). Measurements were made directly from the tail clip. Insulin levels were measured from plasma. Blood was collected from tail clips in lithium heparin Microtainer® tubes (Becton Dickinson), and immediately placed on wet ice until samples from all animals had been collected. Blood samples were spun using a tabletop microcentrifuge (Labnet Spectrafuge™ 16M) for 5 min at 14,000 rpm. Plasma was removed from the samples and frozen at -80 °C until insulin levels could be determined using Ultra sensitive mouse insulin ELISA kits (Crystal Chem, Inc., Chicago, IL). Assays were run according to the manufacturers' protocol. All samples were run in duplicate.

Glucose Tolerance Test

For the glucose tolerance test (GTT) mice were fasted for 6 hrs prior to the start of the test. As determined by their assigned treatment group, mice were given their fourth and final injection of either vehicle or NAX-5055 five hours after the start of the food fast and 1hr prior to the beginning to the test, so that the GTT would begin at the TPE for NAX-5055 (i.e., 1 hr). At the beginning of the GTT (corresponding to time point 0), blood samples were collected for insulin measurement and glucose level determination. Immediately following the collection of these samples mice were administered a 1 g/kg i.p. injection of D-glucose (Sigma-Aldrich, St. Louis, MO). Glucose levels were sampled

at 0, 5, 15, 30, 60, and 120 min post glucose injection. For insulin level determination, blood samples were drawn at 0, 15, 30, and 60 min postglucose injection (Figure 4.1). All mice were immediately sacrificed after the final blood samples were obtained.

Data Analysis

All data and results are reported as means ± standard error of the mean (SEM). Statistical significance was determined by unpaired t-tests when comparing only two groups (Figure 4.2) and one-way ANOVA when comparing three or more groups (Figures 4.3 & 4.4). To determine significance between groups involving multiple comparisons, Bonferroni post hoc tests were used to compare significance between all groups and Dunetts post hoc tests were used to compare experimental treatments to vehicle controls. Area under the curve (AUC) was calculated, using the trapezoid rule, for individual curves generated during the time course of the GTT for both glucose and insulin measurements. A one-way ANOVA of the calculated AUC values was used to determine any significant differences. All statistics were done using GraphPad Prism Software (La Jolla, CA).

Results

Glucose Response to NAX-5055

We have previously demonstrated that a 4 mg/kg systemic injection of the GalR1 preferring galanin analog NAX-5055 protects against acute seizures in the 6 Hz (32 mA)

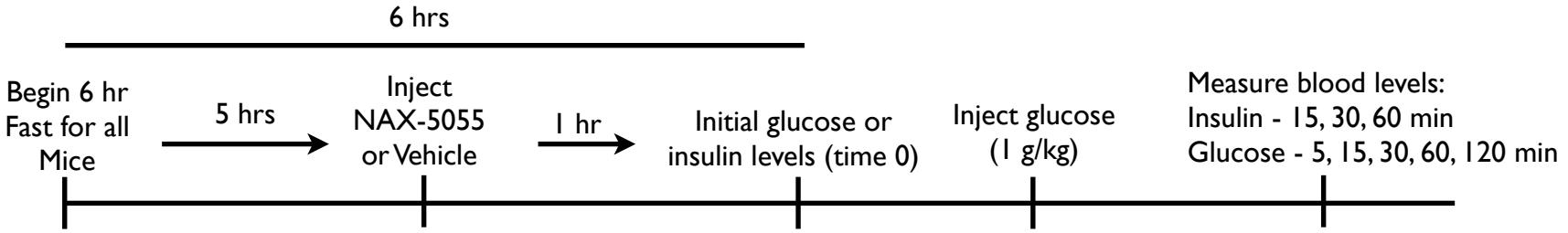


Figure 4.1. Schematic representation of the time course of events for the glucose tolerance test

psychomotor seizure model with minimal motor impairment (Bulaj et al., 2008; White et al., 2009). To determine if this anticonvulsant dose of NAX-5055 would also elicit a physiological response from peripheral pancreatic galanin receptors we measured blood glucose levels 1hr after a single systemic injection of vehicle or NAX-5055 (4 mg/kg). Mice administered NAX-5055 displayed increased glucose levels (mean ± SEM: vehicle, 188.2 ± 5.3 mg/dL; NAX-5055, 312.9 ± 27.9 mg/dL, $p < 0.0001$; n=14, per group) (Figure 4.2A).

To determine if the observed increase in glucose levels following systemic administration resulted from the interaction of the active Gal(1-13) fragment of NAX-5055 with galanin receptors, or as a side effect of the chemical modifications added to increase bioavailability we designed and synthesized a scrambled peptide version of NAX-5055; i.e., 805-1. This peptide was designed with the residues Trp^2 and Tyr^9 reversed. The remaining 11 amino acids of the active fragment and other modifications remained unaltered. These two amino acid residues are known to be critical for high affinity binding of galanin to its receptors (Land et al., 1991; Kask et al., 1998; Lundström et al., 2005). Time-resolved fluorescent binding studies with 805-1 confirm that this peptide does not bind with high affinity to either GalR1 or GalR2 receptors (data not shown). No significant increase in blood glucose levels was observed in mice treated with 805-1 (4 mg/kg) 1 hr postadministration (mean ± SEM: vehicle, 169.8 ± 8.3 mg/dL; 805-1, 164.5 ± 14.6 mg/dL, $p = 0.76$; $n = 4$ per group) (Figure 4.2B). These results suggest the direct interaction of the active galanin fragment of NAX-5055 with the pancreatic galanin receptors is critical for eliciting the hyperglycemic effect observed in mice following NAX-5055 administration.

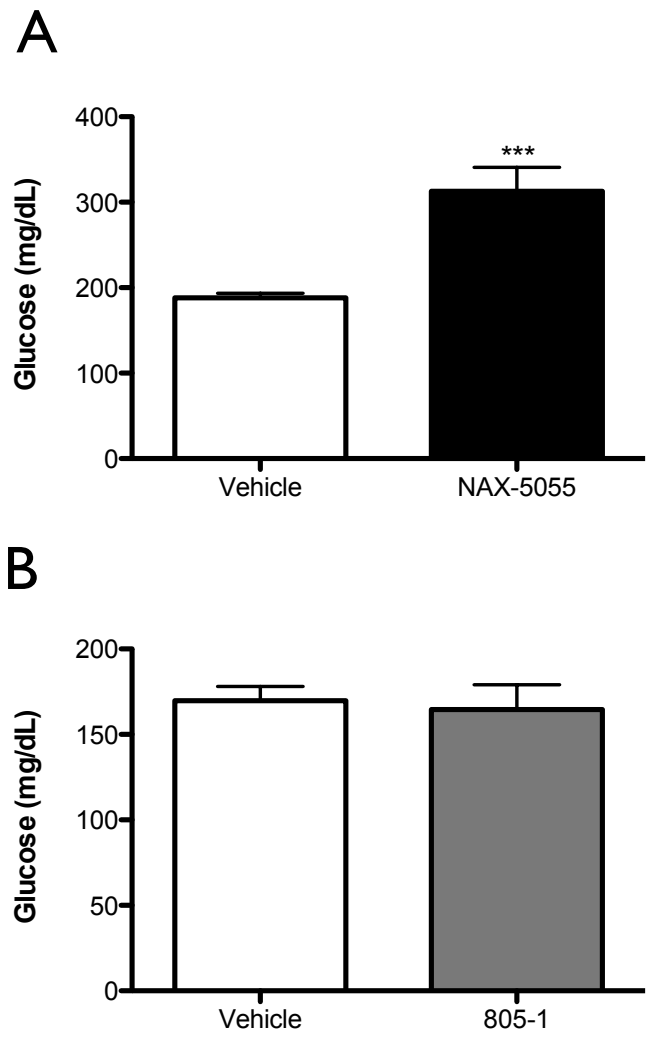


Figure 4.2. Systemic administration of the galanin analog NAX-5055 produces hyperglycemia.

A.) Mice were given a single i.p. injection of NAX-5055 (4 mg/kg) and glucose levels were measured 1 hr after injection. Treated mice showed a significant increase in blood glucose levels (unpaired t-test, ***$p < 0.001$, $n = 8$).

B.) Mice were given a single i.p. injection of the inactive analog 805-1 (4 mg/kg), which displays no affinity for the galanin receptors GalR1 or GalR2. 805-1 did not produce a significant change in blood glucose levels 1 hr after i.p. injection (unpaired t-test, $p > 0.05$, $n = 4$).

Glucose Response to Repeated NAX-5055

Both the GalR1 and GalR2 receptors have been shown to rapidly internalize following chronic agonist exposure *in vitro*. Following internalization, GalR1 receptors are transported to lysosomes for degradation, resulting in decreased membrane expression (Wang et al., 1998; Xia et al., 2005; 2008). To determine if repeated activation of galanin receptors *in vivo* would attenuate the observed hyperglycemia, we repeatedly dosed mice with NAX-5055 and evaluated blood glucose levels one hour after the last challenge dose. For this study, four treatment groups were employed as outlined in Figure 4.3A and described in detail in the Methods section. Mice given either a single or repeated administration of NAX-5055 showed elevated glucose levels compared to vehicle-treated mice (mean ± SEM: vehicle = 157.5 ± 6.5 mg/dL; acute NAX-5055 = 353.5 ± 39.9 mg/dL; repeated NAX-5055 = 378.0 ± 20.8 mg/dL, $p < 0.0001$, n = 8 per group) (Figure 4.3B). In addition, mice repeatedly treated with 805-1 followed by a single administration of NAX-5055 also displayed elevated glucose levels compared to vehicle treated mice (mean ± SEM: vehicle = 157.5 ± 6.5 mg/dL; 805-1 = 297.5 ± 39.0 mg/dL, n = 8, per group) (Figure 4.3B). There was no significant difference between the glucose levels of the acute, repeated or scrambled peptide treatment groups ($p > 0.5$) (Figure 4.3B). These data suggest that repeated exposure of pancreatic galanin receptors to the agonist NAX-5055 *in vivo* does not diminish the hyperglycemia associated with acute NAX-5055 administration.

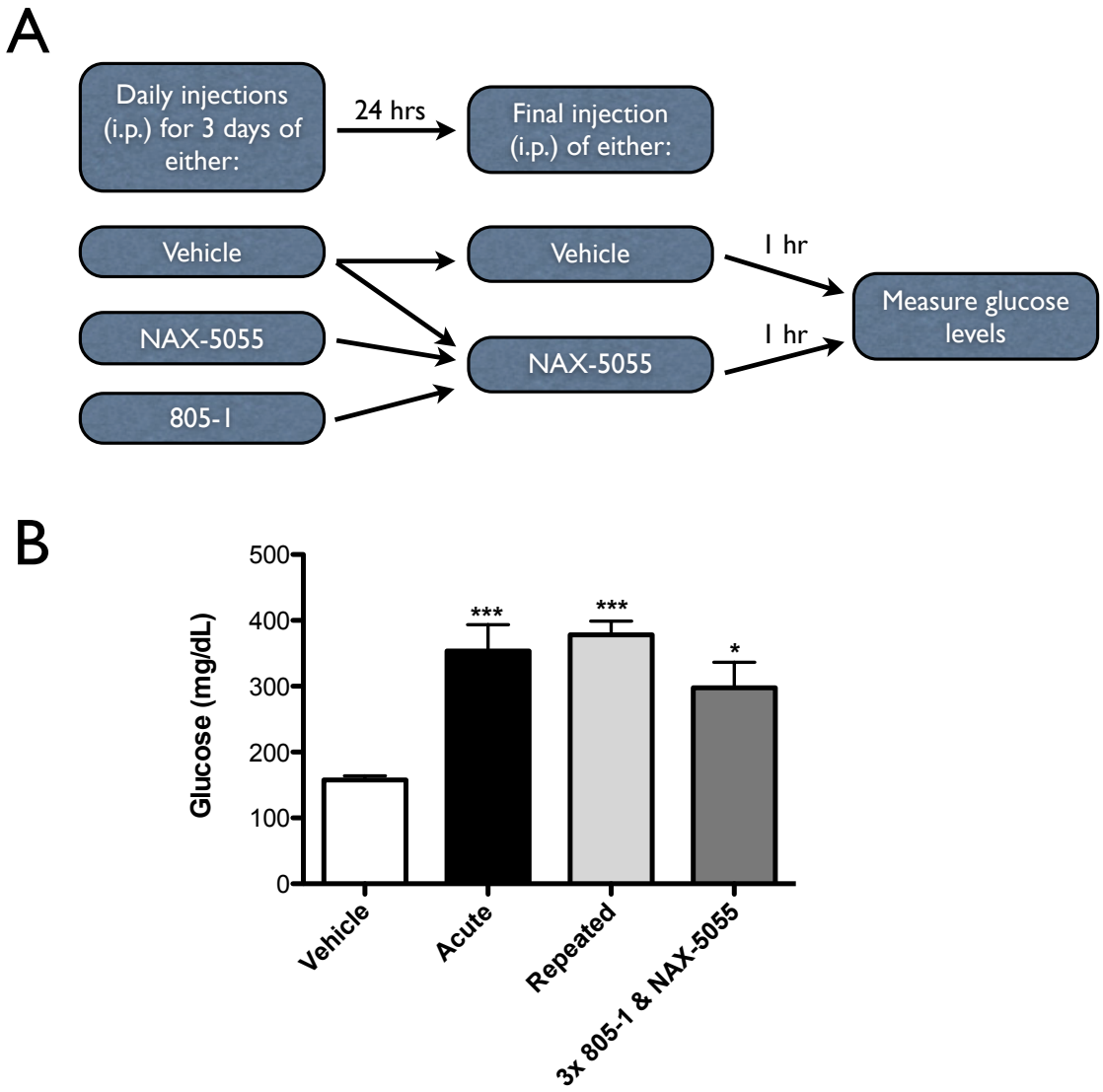


Figure 4.3. Repeated treatment with NAX-5055 does not attenuate the hyperglycemic effect seen with NAX-5055.

A) Schematic of the experimental design.

B) Glucose levels following acute or repeated treatment with NAX-5055 or 805-1; groups described in detail in Methods. All groups that received at least one injection of NAX-5055 showed a significant increase in blood glucose levels (one-way ANOVA, ***$p < 0.001$, *$p < 0.05$, $n = 8$ per group).

Glucose Tolerance Test Following NAX-5055 Administration

Based on the robust hyperglycemia induced by NAX-5055 under basal conditions, we sought to further investigate the effect of NAX-5055 on glucose and insulin levels in response to a glucose tolerance test (GTT). Both acute and repeated administration studies were run in tandem to determine if repeated receptor activation would influence regulation of glucose and insulin levels. For this study three treatment groups were employed as outlined in Figure 2.4A and as described in detail in the Methods section.

We first measured glucose levels in mice treated with either acute or repeated doses of NAX-5055. At the 0 min time point (immediately before glucose injection) both the acute and repeatedly treated mice displayed elevated glucose levels compared to vehicle controls; however a significant differnce was only observed between the vehicle and repeated treatment groups (mean ± SEM: vehicle = 152.8 ± 5.3 mg/dL; acute = 215.5 ± 32.7 mg/dL; repeated = 290.8 ± 28.1 mg/dL, $p = 0.0051$) (Figure 4.4B). All mice showed increased blood glucose levels following glucose injection, but varied in their time to peak effect and the magnitude of the hyperglycemia observed. For example, in vehicle-treated animals glucose levels peaked at 15 min after glucose injection and returned to baseline after 60 min. However, in both the acute and repeated treatment group, glucose levels increased immediately after glucose injection, but did not reach peak levels until after 60 min (Figure 4.4B). Total area under the curve of the glucose response was significantly increased for acute and repeated treatment groups compared to vehicle treated controls ($p < 0.001$). No significant difference in glucose levels between mice subjected to acute or repeated dosing with NAX-5055 was observed (Figure 4.4C).

Figure 4.4. Mice given a single acute or repeated treatment with NAX-5055 show exaggerated hyperglycemia and decreased insulin secretion following a systemic glucose challenge.

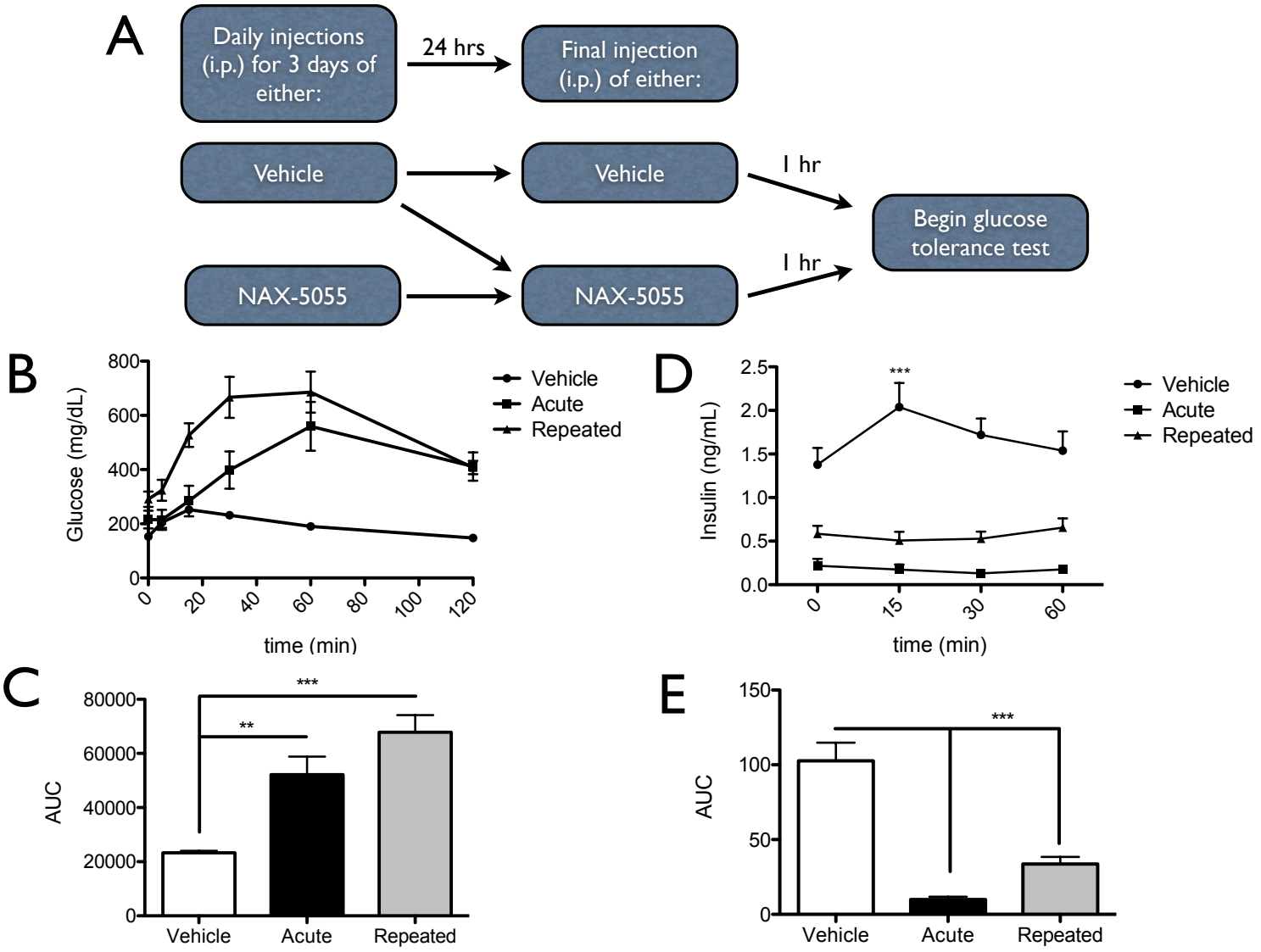
A) Schematic of the experimental design.

B) Glucose levels in vehicle (n = 6), acute (n = 6), and repeatedly (n = 8) treated mice across the duration of the glucose tolerance test.

C) AUC measurements for all groups demonstrate both acute and repeated treatment groups have significantly increased blood glucose levels compared to vehicle treated mice (One-way ANOVA of AUC, **$p < 0.01$, ***$p < 0.001$).

D) Insulin levels in vehicle (n = 16), acute (n = 16), and repeatedly (n = 16) treated mice across the duration of the glucose tolerance test.

E) AUC measurements for all groups demonstrate both acute and repeated treatment groups have significantly decreased plasma insulin levels compared to vehicle treated mice (One-way ANOVA of AUC, ***$p < 0.001$).


A
Daily injections (i.p.) for 3 days of either:
24 hrs
Final injection (i.p.) of either:
Vehicle
Vehicle
1 hr
Begin glucose tolerance test
NAX-5055
NAX-5055
1 hr
B
Glucose (mg/dL)
time (min)
Vehicle
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Given that the direct action of galanin on glucose regulation is proposed to be related to its ability to inhibit insulin release, we measured insulin levels following either acute or repeated treatment with NAX-5055. At time point 0 in the GTT (immediately before glucose injection) both the acute and repeated treatment groups displayed significantly decreased insulin levels compared to vehicle controls (mean ± SEM: vehicle = 1.4 ± 0.19 ng/mL; acute = 0.22 ± 0.08 ng/mL; repeated = 0.58 ± 0.09 ng/mL, $p < 0.001$, n = 16 per group) (Figure 4.4D). Following glucose injection, only the vehicle treated animals showed any glucose-induced increase in insulin levels; insulin levels peaked at 15 min and rapidly returned to preglucose injection levels. The total area under the insulin response curve demonstrates a significant decrease in insulin release for both the acute and repeated treatment groups compared to vehicle treated controls ($p < 0.001$) (Figure 4.4E). In contrast, there was no significant difference between acute and repeated groups ($p > 0.05$).

Discussion

This study is the first to demonstrate that a systemically available GalR1-preferring galanin analog can regulate glucose and insulin levels *in vivo*, in a manner similar to that proposed for endogenous galanin (McDonald et al., 1994; Ahrén, 2000). Acute administration of NAX-5055 resulted in a significant increase in glucose levels under both basal conditions and following an acute glucose challenge. In contrast, insulin levels were significantly reduced following acute NAX-5055 administration under basal conditions. Furthermore insulin levels of NAX-5055 treated mice did not fluctuate in response to an acute glucose administration. The effects of NAX-5055 on both

glucose and insulin levels persisted following a 4-day repeated dosing regimen. This effect is likely mediated by the direct interaction of NAX-5055 with pancreatic galanin receptors.

The observed increase in basal glucose levels following a single administration of NAX-5055 is consistent with previous studies showing hyperglycemia in canine, rat and mouse following intravenous galanin infusion and stimulation of mixed pancreatic nerves, which is associated with galanin release (McDonald et al., 1985; Manabe et al., 1986; Lindskog and Ahrén, 1987). The effect of galanin on glucose levels has largely been attributed to the inhibition of insulin release from β-cells of the pancreas (Gregersen et al., 1991). Our results support this hypothesis as insulin levels are also significantly reduced following acute NAX-5055 administration. Some suggested mechanisms for the inhibition of insulin by galanin include opening of ATP–sensitive K^+ channels, reduction in cAMP levels by inhibion of adenylyl cyclase and direct inhibition of exocytosis (de Weille et al., 1988; Sharp, 1996). Activation of ATP-sensitive K^+ channels and inhibition of cAMP levels has also been seen in the central nervous system following galanin receptor activation and is suggested to play a role in inhibiting neurotransmitter release (Zini et al., 1993; Hawes et al., 2006). It should be noted that intravenous galanin administration not only affects insulin secretion, but it may also increase glucagon levels as seen in both the canine and mouse, but not in rat (Dunning et al., 1986; Lindskog and Ahrén, 1987; Silvestre et al., 1987). During the GTT, both acute and repeated NAX-5055 treated mice showed increased glucose levels; however those receiving repeated NAX-5055 injections displayed higher glucose levels, particularly at the 15 and 30 min time points. Although we did not directly measure glucagon levels in this study it is

tempting to speculate that the robust increase in glucose following administration of NAX-5055 may result from both an inhibition of insulin as well as an increase in glucagon secretion. In addition, mRNA expression levels for the GalR1 receptor are the highest of all three galanin receptors on the β cells (Barreto et al., 2009); thus direct activation of this receptor by NAX-5055 suggests that GalR1 may play a primary role in mediating the effects of galanin on glucose and insulin levels.

The finding that there is no significant attenuation of the hyperglycemic response to NAX-5055 following repeated treatment is particularly interesting. In the past, these types of studies have been difficult to perform with native neuropeptides *in vivo* because of their propensity for rapid degradation following systemic administration (Bulaj et al., 2008). Work with immortalized cell lines *in vitro* has suggested that both GalR1 and GalR2 receptors are prone to internalization following prolonged agonist exposure (Wang et al., 1998; Xia et al., 2004; 2008). Further, internalized GalR1 receptors have been shown to be trafficked to lysosomes for degradation and GalR1 activation-dependent inhibition of cAMP has been suggested to decrease receptor expression (Hawes et al., 2006; Xia et al., 2008). However, in our *in vivo* studies with mice, the hyperglycemic response elicited with single administration of NAX-5055 showed no attenuation to the response seen following four days of repeated dosing. These results suggest that peripheral galanin receptors are responsive to NAX-5055 following both acute and repeated exposure. This activity is dependent on the interaction of NAX-5055 with galanin receptors given that repeated treatment with the scrambled analog 805-1, which does not bind with galanin receptors, did not modify the NAX-5055-induced hyperglycemic response. A limited supply of 805-1 prevented us from assessing whether

repeated treatment with this peptide would directly affect glucose levels. However, we have shown that 805-1 has no effect on glucose levels following acute administration and that repeated administration does not attenuate or exaggerate the hyperglycemic response associated with acute NAX-5055 administration. Thus, it seems likely that repeated treatment with 805-1 would have no effect on glucose levels following repeated administration.

C57/Bl6J mice are often used to test the activity of compounds in the glucose tolerance test (Dunning et al., 1986; Zorrilla et al., 2007). For this study we chose to use the CF-1 mouse in order to directly relate our findings to the anticonvulsant studies that we have previously conducted with NAX-5055 using this mouse strain (Bulaj et al., 2008). Similar to work done with the C57Bl6J mice, glucose administration in vehicle-treated CF-1 mice elevated glucose and insulin levels rapidly; i.e., both peaked within 15 min of glucose injection. Both glucose and insulin levels returned to baseline levels by the completion of the test, confirming glucose sensitivity in this strain of mice. In contrast, the glucose tolerance test conducted in the acutely and repeatedly treated NAX-5055 mice demonstrated an altered response to the glucose challenge, consistent with previous GTT studies with native galanin (Dunning et al., 1986; Lindskog and Ahrén, 1987). The proposed mechanism of galanin in this test is the inhibition of insulin release. Here we have shown that NAX-5055 completely abolishes any glucose stimulated increase in insulin release for the duration of the test. These observations suggest that NAX-5055 is an effective and potent inhibitor of insulin release in mice. Although not statistically significant, the insulin levels observed following repeated treatment are slightly higher than those in the acute treatment group. Thus, it is possible that there is a

slight tolerance to the inhibition of insulin release following repeated treatment with NAX-5055; however, further study is required to fully test this hypothesis.

The altered response to the glucose challenge following both acute and repeated NAX-5055 treatment is evident in the persistent increase in blood glucose levels and lack of insulin response following systemic glucose injection. These findings are consistent with other studies showing galanin-induced glucose insensitivity (Ahrén and Lindskog, 1992). However, studies with either the galanin knockout mouse or GalR1 knockout mouse have also shown glucose insensitivity in a glucose tolerance test; i.e., knockout mice displayed impaired insulin secretion and glucose disposal (Ahrén et al., 2004; Zorrilla et al., 2007). It remains unclear why this conflicting response occurs in these knockout strains. One possibility is that galanin and/or the GalR1 receptor has an important role in the development of normal β-cell innervations or physiological function and thus normal cellular activity is compromised in the presence of the mutation. A second possibility is that there are compensatory changes in galanin receptor number or other neuropeptide systems that may alter the response of β-cells to either galanin or glucose stimulation.

The hyperglycemia and inhibition of insulin release observed with NAX-5055 raises concerns about the use of systemically active GalR1 preferring analogs for the treatment of neurological diseases in humans. However, the effect of galanin on glucose and insulin levels in humans is inconsistent. Similar to mice and dogs, humans express galanin in nerves innervating islet cells of the pancreas (Ahrén et al., 1991). Infusion of human galanin during an oral glucose test in normal humans at doses shown to inhibit insulin release in dogs did not change circulating glucose or insulin levels (McDonald et

al., 1994). However, in isolated human islets cells, infusion of porcine galanin significantly inhibited insulin release (Ahrén et al., 1991). These diffences between *in vivo* and *in vitro* studies suggest that galanin's regulation of insulin levels in humans may extend beyond the its direct effects on β-cells.

The regulation of galanin expression is also directly affected by glucose in humans, such that circulating galanin levels positively correlate with increased glucose levels in a oral glucose test in healthy controls (Legakis et al., 2007). Further, adults with type-2 diabetes and children with type-1 diabetes mellitus show elevated galanin (Celi et al., 2005; Legakis et al., 2005). Thus there is not a direct correlation between the effects of galanin in animal model systems and in humans suggesting the effects we report here for NAX-5055 may not translate to humans. In addition, it is still unclear how each galanin receptor mediates the effects of galanin on β-cell function. The data shown here suggest that GalR1 is directly involved in regulating glucose and insulin levels; however, a non-GalR1 agonist may not display this same effect. Indeed preliminary evidence with GalR2-preferring analogs in our lab demonstrates that targeting this receptor results in anticonvulsant activity without negatively affecting blood glucose levels (unpublished results). Increased understanding of the role of each galanin receptor in the different physiological functions of galanin in both the peripheral and central nervous systems will help mitigate concerns about the clinical use of galanin-based theraputics.

This study is the first to show that a GalR1-preferring galanin analog acts in the endocrine pancreas in a way similar to galanin infusions *in vitro*. Evidence that β-cells contain mRNA to encode all three galanin receptor subtypes enhances the need to further understand the role of each receptor subtype in mediating the neuromodulatory function

of galanin *in vivo*. In conclusion, we have shown here that the GalR1 receptor agonist NAX-5055 is a potent regulator of both glucose and insulin in the mouse under basal and glucose-stimulated conditions. NAX-5055 thus provides the scientific community with a useful pharmacological tool to increase our understanding of how the different galanin receptor subtypes mediate the activity of galanin.

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CHAPTER 5

DISCUSSION

Background and Significance

Epilepsy is one of the most common neurological disorders, affecting patients with no clear bias for age, gender, or socioeconomic status (Institute of Medicine, 2012). The myriad of epilepsy syndromes can manifest in various behavioral phenotypes; however, all patients share the same clinical hallmark of recurrent spontaneous seizures. Although 70% of epilepsy patients achieve seizure freedom with pharmacological treatment, the remaining 30% of patients demonstrate resistantance to pharmacological intervention (Stephen and Brodie, 2012). It has been proposed that in order to effectively treat drug-resistant patients, future research must focus attention on the modulation of the pathological brain via novel molecular targets (Löscher and Schmidt, 2011).

Potential novel targets for the treatment of epilepsy include the neuropeptide galanin and its receptors. Galanin expression is increased following seizure activity (Mazarati et al., 1998; Wilson et al., 2005) and exogenous injection produces antiseizure activity in a number of animal models (Lerner et al., 2008). However, our understanding of the antiseizure properties of galanin has been limited by the lack of receptor-selective and systemically available pharmacological tools. The work presented in this dissertation focuses on characterizing a novel, metabolically stable galanin analog NAX-5055.

Although the results of our studies identify properties of NAX-5055 that minimize its therapeutic potential as an antiseizure medication, NAX-5055 remains a useful experimental tool to study the function of galanin receptors. In addition, the approach and assays discussed in this dissertation provide a useful outline for the future development of neuropeptide-based theraputics.

Summary and Conclusions

The studies described in Chapter 2 were aimed at elucidating the mechanism of action of NAX-5055 in the hippocampus. We hypothesized that NAX-5055, like many other neuropeptides, would reduce hippocampal excitability by inhibiting presynaptic neurotransmitter release (Tallent, 2008). Our studies demonstrate that NAX-5055 increases the inter-event interval of mEPSCs in mouse organotypic hippocampal slice cultures (OHSC) without affecting the amplitude of these events in CA3 pyramidal neurons. These results suggest that NAX-5055 reduces the probability of presynaptic glutamate release onto CA3 pyramidal cells. This is a particularly interesting finding given the preference of NAX-5055 for GalR1 receptors. Confirmation of this finding in future studies would provide the first evidence of a GalR1-mediated presynaptic mechanism of action for galanin.

Acute systemic administration of NAX-5055 displays potent anti-seizure efficacy in a battery of seizure and epilepsy animal models (Bulaj et al., 2008; White et al., 2009). The initial aim of this dissertation was to evaluate the disease modifying potential of NAX-5055 in the corneal kindled mouse. This aim was based on the observation that overexpression of galanin in the brain could delay the development of generalized stage

4/5 seizures in mouse and rat kindling models (Kokaia et al., 2001; Kanter-Schlifke et al., 2007). As described in Chapter 3, NAX-5055 had no effect on the corneal kindling acquisition rate in mice. This was an unexpected finding given the previous literature and led to the hypothesis that the failure of NAX-5055 to alter kindling acquisition could result from a reduction in the efficacy or potency of NAX-5055 following repeated systemic administration. To test this hypothesis, mice were administered systemic injections of NAX-5055 (4 mg/kg) once daily for four consecutive days and then tested in the 6Hz psychomotor seizure model. The antiseizure efficacy of NAX-5055 was dramatically reduced following repeated systemic injection, suggesting a reduction in the efficacy of NAX-5055 at this dose. Similar effects were observed in C57BL/6J mice and fully kindled CF-1 mice, suggesting this effect is not strain or model specific.

The results of these repeated dosing experiments shifted the focus of this dissertation work to studies that aimed to define the mechanism(s) responsible for the reduced efficacy of NAX-5055 observed following multiple doses. I proposed three independent hypotheses that could account for the observation of this phenomenon. The first hypothesis is the "target hypothesis," which proposes that drug targerts are altered following repeated exposure. The second hypothesis is the "transport hypothesis," which proposes that drug transport is altered following repeated exposure. Both of these hypotheses have been found to explain the development of drug-resistance in epilepsy patients (Remy and Beck, 2006; Kwan and Schachter, 2011). The third hypothesis, discussed in Chapter 4, proposes that peripheral metabolism of NAX-5055 is increased, which would result in a decrease in the bioavailability of functional peptide.

The Target Hypothesis

The "target hypothesis" of drug-resistant epilepsy proposes that alterations to the cellular targets of antiseizure drugs results in a reduction of their sensitivity to pharmacological engagement (Remy and Beck, 2006; Kwan and Schachter, 2011). A clinical example supporting this hypothesis comes from studies conducted with hippocampi resected from patients resistant to the antiseizure effects of carbamazepine. Electrophysiological recordings demonstrated a lack of use-dependent block of Na^+ channels normally observed in the presence of carbamazepine as well as an inability to block induced seizure activity in the slice (Remy et al., 2003).

The target hypothesis appeared viable to explain the reduction in efficacy of NAX-5055; particularly given that galanin receptors are prone to internalization following agonist exposure *in vitro* (Wang et al., 1998; Xia et al., 2005; 2008). Further, the internalization and trafficking of peptide G-protein-coupled receptors has been extensively studied, and is commonly proposed as a mechanism to explain observed reductions in drug efficacy (Bernard et al., 2006; Kelly et al., 2008). To evaluate the reduction in efficacy of NAX-5055 we generated dose response curves for mice given either acute or repeated doses of NAX-5055. When all doses were administered systemically, we observed a significant rightward shift in the dose-response curve for repeatedly treated mice (Figure 3.5). This response shows that galanin receptors are available for interaction with NAX-5055 following repeated treatment, but increased concentrations are required to achieve the same efficacy. This suggests a decrease in potency of NAX-5055 following repeated systemic injection. In contrast, no significant shift in dose-response curves was noted when the final dose of NAX-5055 was

administered directly into the ventricular space of the brain. This observation is consistent with the hypothesis that there is no change in galanin receptor availability following repeated systemic injection.

The Transport Hypothesis

The "transport hypothesis" of drug-resistant epilepsy proposes that an increase in the expression of drug-efflux transporters results in a reduced concentration of a anti-seizure drug in the brain (Remy and Beck, 2006; Kwan and Schachter, 2011). Evidence for this hypothesis comes from studies showing that the ATP-binding cassette transporter p-glycoprotein is upregulated in the capillaries of hippocampal tissue resected from temporal lobe epilepsy patients (Kwan et al., 2010). In addition, *in vitro* studies have shown that a number of antiseizure drugs can act as substrates for p-glycoprotein (Tishler et al., 1995; Luna-Tortós et al., 2010). To our knowledge, there is no known transport mechanism for galanin within the central nervous system. However, NAX-5055 has a number of chemical modifications to its galanin core that could make it susceptible to active efflux transport. To determine if efflux transport of NAX-5055 could account for the reduced potency of NAX-5055 observed following repeated systemic injection, we evaluated whether it could serve as a substrate for mouse p-glycoprotein. In Chapter 3 we show that NAX-5055 does not interact with either isoform (mdr1a and mdr1b) of mouse p-glycoprotein. Thus, it seems unlikely that efflux transport through p-glycoprotein is suitable to explain the observed reduction in potency of NAX-5055.

Peripheral Metabolism of NAX-5055

The third and final hypothesis evaluated in an effort to explain the reduced potency of NAX-5055 proposes that an increase in peripheral metabolism would reduce the amount of functional peptide available for interaction with galanin receptors. The most direct approach to address this hypothesis would be to compare plasma and brain concentrations of NAX-5055 following acute and repeated systemic administration. However, we have not been able to determine brain or plasma levels of NAX-5055 using analytical chemistry techniques. To circumvent this issue, we took an indirect approach and tested the activity of NAX-5055 on peripheral galanin receptors. Galanin receptors are expressed on β-cells of the pancreas and activation of these receptors has been shown to inhibit insulin release (Tatemoto et al., 1983; Lindskog and Ahrén, 1987; Barreto et al., 2011). In Chapter 4 we show that NAX-5055 significantly reduces plasma concentrations of insulin following a single systemic administration. There was no attenuation of this effect in mice subjected to repeated systemic NAX-5055 injections. The lack of attenuation in the insulin response suggests that NAX-5055 remains in an active form that can engage galanin receptors following repeated administration. In summary, our studies were unable to describe a clear mechanism for the reduced potency of NAX-5055 following repeated systemic administration.

Speculations and Future Directions

Throughout this dissertation work we have often made the assumption that NAX-5055 exerts its activity largely through activation of the GalR1 receptor. This assumption is based on binding studies demonstrating that NAX-5055 has a 15-fold preference for

the GalR1 receptor over the GalR2 receptor (Bulaj et al., 2008). However, NAX-5055 maintains nanomolar affinity for GalR1 and GalR2; as such, NAX-5055 could engage either receptor subtype following systemic and/or central injection. This is of particular relevance given that it is unclear what the levels of NAX-5055 are within the brain following systemic administration. In an effort to test this assumption we have recently acquired and genotyped GalR1 knockout mice (GalR1-KO). These mice display minimal physiological differences from wild-type mice; further, they show no alteration in the expression of GalR2 or GalR3 receptors (Jacoby et al., 2002; McColl et al., 2006; Schauwecker, 2010). Evaluation of the anti-seizure activity of NAX-5055 in these mice will be essential in confirming if GalR1 is the functional molecular target of NAX-5055 within the brain.

It will also be interesting to determine the seizure thresholds of GalR1-KO mice in acute behavioral seizure models. To date, GalR1-KO mice have only been tested in status epilepticus (SE) models. In these studies GalR1-KO mice demonstrate increased susceptibility to both perforant path- and Li-pilocarpine induced SE (Mazarati et al., 2004). We would hypothesize that GalR1-KO mice will display decreased seizure thresholds in mouse seizure and epilepsy models. It is also tempting to speculate that GalR1-KO mice will display decreased seizure thresholds in the partial seizure models in which NAX-5055 displays anti-seizure activity (e.g., 6 Hz psychomotor seizure & the kindling models). This observation would further support the hypothesis that the antiseizure efficacy of NAX-5055 is GalR1-dependent. Together, positive results in these studies would provide further validation of the GalR1 receptor as a CNS target for antiseizure drug development.

Changes in galanin receptor expression and function within the brain following repeated agonist exposure remains an interesting question that we are now well positioned to address. To date galanin receptor regulation has only been studied in immortalized cell lines (Xia et al., 2005; 2008). In the present work, when a challenge dose of NAX-5055 was administered into the lateral ventricle, dose-response experiments failed to conclusively demonstrate a difference in seizure protection between acute or repeatedly treated mice. However, we were unable to reach 100% seizure protection with any dose of NAX-5055 administered intracerebroventricularly. This observation could result from off-target effects at the higher doses of NAX-5055 that may limit our ability to directly determine the effect of galanin receptor activation on seizure susceptibility. As a result, it is unclear if the highest doses of NAX-5055 administered were sufficient to saturate all available galanin receptors. Thus, it remains possible that there are subtle changes in central galanin receptors following repeated agonist administration that cannot be detected with this experimental approach. Even small changes in the expression of G-protein-coupled galanin receptors could have meaningful physiological effects due to the activation of robust intracellular signaling cascades.

One additional approach that could be employed to test for changes in central galanin receptor activity *in vivo* is to repeatedly deliver NAX-5055 directly to the brain via implanted cannula and osmotic pumps. If galanin receptors are indeed unaltered by repeated agonist exposure we would hypothesize that, under these conditions, the antiseizure activity of NAX-5055 would be maintained for the duration of release from the implanted osmotic pumps. This hypothesis is supported by the observation that the antiseizure activity is maintained when galanin is overexpressed and constitutively

released by adeno-associated viral vectors (Haberman et al., 2003; McCown, 2006; Foti et al., 2009). In contrast, studies in immortalized cell lines suggest galanin receptors are internalized following agonist exposure and that GalR1 receptors are rapidly degraded following internalization (Xia et al., 2005; 2008). This discrepancy underlies the importance of testing receptor regulation using both *in vivo* and *in vitro* approaches (Koenig and Edwardson, 1996; Coutts et al., 2001).

To complement the proposed *in vivo* studies, we are developing an *in vitro*, time resolved fluorescence, saturation-binding assay utilizing europium-labeled galanin, specific for isolated brain tissue. This assay will allow us to directly measure the concentration of galanin receptors in the hippocampus of mice subjected to either acute or repeated NAX-5055 injection. Coincubation of tissue preparations with unlabeled galanin and Gal(2-11) will allow us to identify any alterations in the concentration of GalR1, compared to GalR2, following NAX-5055 treatment. One caveat of this approach is that it does not allow us to distinguish cell type specificity, which could be achieved utilizing histological techniques. Unfortunately, a number of commercially available galanin receptor antibodies have been found to lack specificity for their targets; thus limiting our ability to conduct histological studies (Lu and Bartfai, 2009).

The aforementioned binding studies will help to define whether there are any alterations in galanin receptor expression; however, additional studies would be required to evaluate potential changes to the function of these receptors. In Chapter 2 we show that acute exposure of NAX-5055 may reduce hippocampal excitability by reducing presynaptic glutamate release onto CA3 pyramidal neurons. Similar to the behavioral studies, a necessary future direction of this work is to evaluate mEPSCs in hippocampal

slices from GalR1-KO mice to determine if the observed effects of NAX-5055 are indeed GalR1-dependent.

We are also particularly interested in the effects repeated exposure of NAX-5055 will have on the functional regulation of galanin receptors. One advantage of using mouse OHSC is the ability to preincubate slices with NAX-5055 and then record whether the sensitivity of the receptors to NAX-5055 changes in response to continuious agonist exposure. If the results of our central administration dose response curves are predictive, then there should be no change in NAX-5055-induced reduction of glutamate release. However, if galanin receptors are prone to agonist-induced modulation, then preincubation with NAX-5055 should attenuate the observed increase in inter-event interval of mEPSCs. We hypothesize that this attenuation would result from galanin receptor internalization. Expression of fluorescently tagged GalR1 receptors in OHSCs generated from GalR1-KO mice would allow us to optically track receptors following agonist exposure. This approach would be the first to show a correlation between membrane galanin receptor expression and function in neurons.

The use of NAX-5055 along with other galanin receptor preferring analogs will also allow us to further elucidate the role of galanin receptors in regulating synaptic transmission. The studies discussed in this dissertation focused on spontaneous glutamate release; however, galanin receptors are also likely to play important roles in the regulation of action potential mediated vesicular release within the hippocampus. In myenteric neurons and the nucleus tractus solitarius galanin has been demonstrated to inhibit voltage-dependent calcium channels though a GalR1-dependent mechanism (Endoh et al., 2008; Anselmi et al., 2009). In contrast, studies in dissociated dorsal root

ganglion neurons demonstrated that galanin enhanced calcium currents via a GalR2-dependent mechanism (Kerekes et al., 2003). These galanin receptor-mediated alterations in calcium concentrations would be expected to increase or decrease cell excitability and thus alter the probability of spontaneous and/or evoked vesicular release. Thus a further extension of the work presented in Chapter 2 would be to characterize the role of GalR1 and GalR2 in the spontaneous and evoked release of neurotransmitters within the hippocampal formation.

In addition to potential modification of galanin receptors, the transport hypothesis remains a potential mechanism to explain the loss of potency observed with repeated NAX-5055 administration. The dose-response curves following systemic injection suggest that the efficacy of NAX-5055 is unaltered, whereas potency is reduced. This observation could be explained by altered transport mechanisms. Transport of NAX-5055 away from central galanin receptors would reduce the likelihood of ligand-receptor interaction. This effect could be overcome by increasing the available concentration of NAX-5055 as a result of increasing the administered dose. This speculation is confounded by the fact that there are no known transporters that target neuropeptides in the brain. However, we cannot rule out the possibility that efflux transport of NAX-5055 away from galanin receptors contributes to the observed reduction in antiseizure potency. Even though our studies suggest that NAX-5055 is not a substrate for p-glycoprotein, there is increasing evidence that additional transporters are modulated in epilepsy and that they may be involved in trafficking xenobiotics, such as NAX-5055. These transporters include the breast cancer resistance protein (BCRP), the multi-drug resistance transporters (Mdr), and non-ABC transporters (Aronica et al., 2011).

Additional screening is thus necessary to determine if NAX-5055 can interact with any of these transporters. If an interaction were identified, it would be of great interest to determine the site of interaction. If the active galanin fragment of NAX-5055 is shown to interact with a transporter, this could open up a new line of research into the role of efflux transport in the central regulation of neuropeptides.

Finally, although not discussed in this dissertation, a side project in the laboratory has been investigating the interaction between NAX-5055 and the antiseizure drug levetiracetam (LEV). Both drugs are thought to exert their antiseizure activity through presynaptic mechanisms (Lee et al., 2009). Interestingly, they also display similar anti-seizure profiles in animal seizure and epilepsy models; i.e., they both demonstrate potent activity in models of partial seizure (Klitgaard et al., 1998; Bulaj et al., 2008; White et al., 2009). Preliminary experiments have shown that these drugs display synergy when administered together. In addition, repeated administration of NAX-5055 reduces the efficacy of LEV in the 6 Hz psychomotor seizure model. It is tempting to speculate that these drugs share a similar mechanism; e.g., the regulation and response to intracellular Ca^{2+} (Lynch et al., 2004; Anselmi et al., 2009). Study of the interaction of these two drugs could prove interesting in further understanding their mechanism of action.

Taken together, the studies presented in this dissertation were unable to provide a clear mechanism to explain the reduced potency of NAX-5055 following repeated systemic injection. A summary of results with evidence for or against each hypothesis is outlined in Table 5.1. Although we have not exhaustively ruled out any of the three proposed mechanisms, our studies support the conclusion that galanin receptor modulation is unlikely to account for the reduced potency of NAX-5055. The two

Table 5.1 Summary of potential mechanism(s) for reduced potency of NAX-5055 following multiple dosing

Hypotheses for the reduced efficacy of NAX-5055	Support for the hypothesis	Evidence against the hypothesis
Target hypothesis of NAX-5055	• GalR1 & GalR2 receptors are internalized following agonist exposure *in vitro* • Anti-seizure drugs demonstrate reduced efficacy in resected tissue from epileptic patients	• Anti-seizure activity observed with galanin overexpression in mice and rats • NAX-5055 demonstrates a shift in potency, and not efficacy following repeated NAX-5055 dosing • No difference in central administration dose response curves • No change in peripheral galanin receptor response to NAX-5055 following repeated administration
Transport hypothesis of NAX-5055	• Increased P-gp expression in resected tissue from epileptic patients • Anti-seizure drugs can act as substrates for P-gp • Shift in potency of NAX-5055 following repeated administration	• NAX-5055 is not a substrate for mouse P-gp • No known mechanism for active transport of neuropeptides has been identified
Increased Metabolism of NAX-5055	• Peptides are prone to metabolism by peptidases • Unknown pharmacokinetics • Shift in potency of NAX-5055 following repeated administration	• No difference in insulin levels following acute or repeated NAX-5055 administration

studies arguing against the target hypothesis are the repeated administration dose-response studies where the last dose of NAX-5055 was administered peripherally or centrally, and the insulin release studies conducted after acute and repeated NAX-5055 dosing. If alterations in galanin receptors were involved in reducing the potency of NAX-5055 we would have expected an attenuated response following NAX-5055 repeated dosing. However, in both experiments no difference was observed in the behavioral or physiological response of mice that received either acute or repeated doses of NAX-5055. This conclusion is supported by studies that showed antiseizure activity in the presence of constitutively released, overexpressed galanin in mice and rats (Haberman et al., 2003; McCown, 2006). These studies suggest that central galanin receptors are functional in the presence of chronically elevated extracellular galanin levels. The future studies proposed here will help further elucidate a clear mechanism(s) for the reduction in NAX-5055 potency *in vivo*.

Although this work characterizes a number of unfavorable *in vivo* properties of NAX-5055 (e.g., hyperglycemia and reduced efficacy with repeated administration), it has also established a series of new methodologies in our laboratory. These approaches will facilitate the future characterization of novel galanin analogs. Our studies have centered on a GalR1-preferring compound; however additional GalR2-preferring compounds have also been designed and synthesized. It is possible that GalR2-preferring compounds will display similar therapeutic potential without unfavorable side effects. Further, the ability to selectively target GalR1 or GalR2 receptors pharmacologically will enhance our understanding of the function of galanin and its receptors under basal and pathological conditions. In conclusion, the development of an effective galanin-based

analog may prove useful for the treatment of epilepsy and other neurological disorders including neuropathic pain, anxiety disorders, and Alzheimer's disease.

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